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**The Dissertation Committee for Tsan Yu Chiu Certifies that this is the approved
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**The Localization and Biochemical Analysis of *Arabidopsis thaliana*
APYRASE1 through 7**

Committee:

Stanley Roux, Supervisor

Enamul Huq

Arlen Johnson

Mona Mehdy

Randy Linder

**The Localization and Biochemical Analysis of *Arabidopsis thaliana*
APYRASE1 through 7**

by

Tsan Yu Chiu, B.S.; M.S.

Dissertation

Presented to the Faculty of the Graduate School of
The University of Texas at Austin
in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

**The University of Texas at Austin
December, 2012**

Dedicated to

My family:

Dad, Mom, Yu-Chin, Ching-Ying and Yu-Jhan

Acknowledgements

Gaining PhD has been a tough and emotional journey for me. First of all, I would like to thank my supervisor Dr. Roux. Dr. Roux laid out several challenges during this journey which trains me to become a more independent and stronger researcher. He is like the gardener describe in John 15:1-2. ‘I am the true vine, and my Father is the gardener. He cuts off every branch in me that bears no fruit, while every branch that does bear fruit he prunes so that it will be even more fruitful’. Second, I would like to thank all my committee members for their valuable suggestions in my work especially to Dr. Huq. When I was in the pit hole, he was there holding me from falling deeper.

My special gratitude to my co-laborators: Dr. Heazlewood, Dr. Orellana, and Dr. Loqué. Without their help, I may not finish this work especially Dr. Heazlewood. He has provided me with tremendous help, resources and intellectual advices. I would like to thanks all my friends from JBEI especially Katy, Jason, Jeemeng and, Fan for their help when I was in JBEI.

Special thanks to my friends: Jian Wu, Ling, Zhu, Qingyun Bu, and Devin Fraley and friends from Taiwan. When I am emotional, melting down, you are always there. I would like to thank my friend and my TA boss, Stuart Reichler has been always promotive and supportive for me.

Thanks all the supports form my dearest family members. Thanks for my parents and Ching-Ying’s parent easing the stress of raising Yu Jhan and working on my PhD.

Thanks to my husband for his endless love and patience.

The Localization and Biochemical Analysis of *Arabidopsis thaliana*

APYRASE1 through 7

Tsan Yu Chiu, PhD

The University of Texas at Austin, 2012

Supervisor: Stanley Roux, Jr.

NTPDases (Apyrases) (EC 3.6.1.5) require divalent cations (Mg^{2+} , Ca^{2+}) for hydrolysis of di- and triphosphate nucleotides, but do not hydrolyze monophosphate nucleotides. They are insensitive to inhibitors of F-type, P-type, and V-type ATPases and are categorized as E-type ATPases. They are grouped in the GDA_CD39 superfamily.

Seven NTPDases (AtAPY1-7) have been cloned from Arabidopsis. In this work, AtAPY1 or AtAPY2 tagged with C-terminal green fluorescence protein (GFP) and driven by their respective native promoter displayed Golgi apparatus localization. These GFP constructs can rescue the *apy1 apy2* double knockout (*apy1 apy2 dKO*) successfully, which indicates their accuracy and functionality in localization studies. Furthermore, both AtAPY1 and AtAPY2 can complement the *Saccharomyces cerevisiae* Golgi-localized GDA1 mutant by rescuing its aberrant protein glycosylation phenotype. The GFP tagged AtAPY1 or AtAPY2 constructs in the *apy1 apy2 dKO* plants can restore microsomal UDP/GDPase activity in the mutants confirming that they both also have functional competency. Loss-of-function *apy1*, *apy2* and *APY1RNAi apy2* mutants showed higher levels of galactose in the cell wall monosaccharide analysis. However, the efficiency of the galactose transport was not altered *APY1RNAi apy2* mutants.

AtAPY3 through 7 all displayed intracellular localization by transiently expressed C-terminal tagged YFP in the onion epidermal cells. AtAPY3 showed a subcellular localization distinct from the others. Biochemical analyses showed that AtAPY3 prefers

to hydrolyze NTP more than NDP. AtAPY4 resides in the cis-Golgi. It has fairly weak NTPDase activity but can still rescue some part of the phenotypic defects in Golgi luminal NTPDases mutants. AtAPY5 is a strong NDPase and has a broad spectrum of substrate preferences. It can fully restore phenotypic defects in Golgi luminal NTPDases in yeast. AtAPY6 and AtAPY7 are ER and Golgi associated. However, the expression of these two enzymes cannot be detected in the *Saccharomyces cerevisiae* host, which prevents further analysis.

Taken together these results reveal that the current seven APYRASE members are intracellularly associated with Golgi/ER or unknown vesicles. They all display typical NTPDase enzyme activities that can hydrolyze di- or triphosphate nucleotides in the cells.

Table of Contents

List of Tables	x
List of Figures	xi
Chapter 1. Introduction	1
General introduction of GDA1_CD 39 super family.....	1
The biochemical characterization of surface localized mammalian NTPDases	2
Extracellular nucleotide signaling and cell surfaced NTPDases in plant cells.....	4
Plant cell surface localized NTPDases	5
Extracellular matrix synthesis and intracellular NTPDases.....	6
Intracellular localized NTPDase	8
Plant intracellular NTPDase	10
APYRASEs in Arabidopsis	11
Summary	12
Chapter 2. AtAPY1 and AtAPY2 Function as Golgi localized Nucleoside Diphosphatases in <i>Arabidopsis thaliana</i>	17
Abstract	17
Key words	18
Introduction.....	19
Results.....	23
Both AtAPY1-GFP and AtAPY2-GFP can complement <i>apy1 apy2 dKO</i> plants	23
AtAPY1 and AtAPY2 functionally localize to the Golgi apparatus.....	24
AtAPY1 and AtAPY2 are functionally similar to GDA1 of <i>Saccharomyces cerevisiae</i>	25
NDPase activity is reduced in microsomes of Arabidopsis apyrase mutants	26
Matrix polysaccharide composition and UDP-galactose transport in the Arabidopsis apyrase mutants	27

Discussion	28
Conclusion	32
Materials and Methods.....	33
References.....	54
 Chapter 3. The localization and biochemical characterization of <i>Arabidopsis thaliana</i> APY3 through 7	61
Introduction.....	61
Results.....	63
The subcellular localization of AtAPY3-7	63
The heterologous expression of APY3-7 in yeast.....	63
AtAPY5 can rescue the Carboxypeptidase Y glycosylation in <i>Δgda</i> ..	64
None of the AtAPY3-5 can rescue hygromycin sensitivity of <i>Δynd</i> cells	65
Defect in cell growth due to disruption of GDA and YND can be reverted by AtAPY4 and AtAPY5.....	65
AtAPY 3, 4 and 5 show distinct enzyme activities.....	66
Discussion	67
Materials and Methods.....	70
 Chapter 4. Conclusions	88
Appendices.....	92
Bibliography	95

List of Tables

Table 2.1 Monosaccharide composition analysis of wall fractions of apyrase mutants (mol%).	53
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List of Figures

Figure 1.1. The GDA1_CD39 superfamily.....	14
Figure 1.2. Model of how ATP serves as a signaling molecule in the extracellular matrix (ECM) in mammalian cells..	15
Figure 1.3. A model of how ATP may serve as a signaling molecule in the extracellular matrix (ECM) in plant cells..	16
Figure 2.1. Genetic characterization and isolation of <i>apy1apy2</i> dKO plants with either PROAPY1:APY1-GFP or PROAPY2:APY2-GFP constructs.....	39
Figure 2.2 Arabidopsis AtAPY1 and AtAPY2 co-localized with the cis-Golgi marker	41
Figure 2.3 Immunoblot analysis of <i>apy1 apy2 dKO</i> lines containing AtAPY1-GFP and AtAPY2-GFP constructs.....	44
Figure 2.4. Complementation of Golgi-localized yeast apyrases with AtAPY1 and AtAPY2	45
Figure 2.5. Nucleotide diphosphatase activities in the Arabidopsis apyrases mutants.	46
Figure 2.6. Effect of knocking out AtAPY1 and AtAPY2 on monosaccharide composition of cell wall extracts and nucleotide sugar transport.	47
Figure 2.7. Schematic diagrams of AtAPY1 and AtAPY2 loci showing T-DNA insertion sites and C-terminal GFP fusion constructs.....	49
Figure 2.8. RT-PCR of T2 populations to detect native AtAPY1 or AtAPY2 transcript..	50
Figure 2.9. Immunoblot and SDS-PAGE of <i>apy1 apy2 dKO</i> lines containing AtAPY1-GFP and AAPY2-GFP constructs...	51
Figure 2.10. Alignment of primary sequence of AtAPY1 and AtAPY2.....	52

Figure 3.1. Alignment of primary sequence of human NTPDase 1-8 and Yeast GDA1 and YND1 with AtAPY3-7.....	75
Figure 3.2. The subcellular localization of APY3-7 by C-terminal YFP tag.	80
Figure 3.3. The heterologous expression of AtAPY3-5 in various yeast backgrounds.	81
Figure 3.4. The heterologous expression of AtAPY6 and 7 in various yeast backgrounds... ..	82
Figure 3.5. Complementation of Golgi-localized yeast GDA with AtAPY3-5.....	84
Figure 3.6. Complementation of Golgi-localized yeast NTPDase orthologue YND or GDA with AtAPY3-5.	84
Figure 3.7. Genetic characterization and isolation of $\Delta gda\Delta ynd$ dKO yeast....	85
Figure 3.8. Expression Arabidopsis APYRASEs can rescue the slow growth of $\Delta gda\Delta ynd$ dKO....	86
Figure 3.9. Enzyme activity assay of AtAPY3, 4 and 5.....	87
Figure 4.1. Effects of tunicamycin on APYRASE mutant roots.....	92
Figure 4.2. Effects of knocking out APYRASEs on callose deposition in the mutant roots. Bright field (upper panel) and epifluorescence images (bottom panel) of Arabidopsis root tips stained with the callose-specific fluorescent dye aniline blue.....	93
Figure 4.3. Immunoblot analysis of N-link glycosylation markers in APYRASE mutants.....	94

Chapter 1. Introduction

General introduction of GDA1_CD 39 super family

The nucleoside triphosphate diphosphohydrolases are enzymes that hydrolyze both di- and triphosphate nucleotides (NTPDase; NDPase). This type of enzyme requires broad divalent cation (Mg^{2+} , Ca^{2+}) for activation. They are insensitive to F-type, P-type, and V-type ATPases inhibitors. Thus they are grouped as a unique GDA1_CD39 superfamily according to five apyrase conserved regions (ACRs), which is named after two of the best known NTPDases: the yeast GDA1 and the human lymphoid cell CD39 (Knowles, 2011). Currently, nine types of NTPDases (NTPD1-8 and YND) are identified in eukaryotic cells (Knowles, 2011). The members of this type of enzyme are not only can be found on the cell surface (NTPDase type 1 to 3 and 8) but also intracellularly (NTPDase type 4 to 7 and YND1) (Fig. 1.1) and serve distinct functions at these two locations (Knowles, 2011).

Extracellular nucleotide signaling and cell surfaced NTPDases in mammalian cells

Extracellular nucleotide signaling has been investigated extensively in mammalian cells. Various purine and pyrimidine compounds (ATP, ADP, UTP, UDP, UDP-glucose etc.) are known to activate cell surface receptors and mediate signaling pathways inside the cell (Burnstock et al., 2007). The receptors can be categorized into two major types: adenosine (P1) receptors or nucleotide (P2) receptors. The P1 receptors (A1, A2A, A2B, and A3) are G protein-coupled receptors that are activated only by adenosine (ADO). The P2 receptors can act via an ionotropic (P2X) or G protein-coupled (P2Y) manner. The P2X receptors activated by ATP can also channel Na^{+} , K^{+} , and Ca^{2+} across the membrane. The P2Y receptors have more diverse ligands and also couple with G proteins. They include P2Y1 (ADP), P2Y2 (UTP=ATP), P2Y4 (UTP), P2Y6 (UDP), P2Y11 (ATP, NAD^{+}), P2Y12 (ADP), P2Y13 (ADP), and P2Y14 (UDP, UDP-glucose and other nucleotide sugars). In animals, the export of ATPs to the ECM has been demonstrated to be delivered through secretory vesicles (Bodin and Burnstock, 2001a) or channel proteins and plasma membrane-located ABC transporters (Bodin and Burnstock, 2001b; Dutta et al., 2002; Lazarowski et al., 2003) (Fig. 1.2).

Extracellular ATPs play several important physiological roles in mammalian cells. For example, they can function as neurotransmitters in the nervous system to depolarize the membrane and send signals to neighboring neurons (Komoszynski and Wojtczak 1996). Also, they regulate platelet aggregation in blood vessels (Lohman et al., 2012). In these and other signaling functions of eATP, it is important to have tight control of the eATP availability in the ECM to prevent the desensitization of the receptors. This control is exerted largely by four major types of ecto-nucleotidases: E-NTPDase (APYRASEs), ecto-5'-nucleotidase (eN), ecto-nucleotide pyrophosphatase/ phosphodiesterases (E-NPPs), and alkaline phosphatase. These enzymes are jointly responsible for hydrolyzing the eATP so as to maintain the optimal concentration for the purinoreceptors (Robson et al., 2006) (Fig. 1.2).

The biochemical characterization of surface localized mammalian NTPDases

The cell surface-localized NTPDases have two transmembrane domains (TMDs) at their N- and C- termini and ten cysteine residues. They also share more similarities in the additional four conserved regions (CR) than the intracellular members (Fig. 1.3). They regulate the extracellular nucleotide availabilities for the nucleotide (P2) receptors or adenosine (P1) receptors. The following will give details about the current biochemical characterization of these four members.

NTPDase1

In animal cells, NTPDase 1 (CD 39) is the best known cell surface-localized NTPDase. The enzyme has similar activities in the presence of Ca^{2+} or Mg^{2+} and its optimal pH is at pH 7.0-7.5 with ATP and pH 7.5-8.0 with ADP (Kukulski et al., 2005; Chritoforidis et al., 1995). The two TMDs are necessary for the catalytic activity and substrate specificity, because removing both TMDs will abolish 90% of the enzyme activity (Grinthal and Guidotti, 2002; Wang et al., 1998). The physiological significance of NTPDase 1 is that it regulates platelet aggregation by terminating signal transduction initiated by nucleotides such as extracellular ATP (eATP) (Gyle et al., 1999; Buergler et al., 2005).

NTPDase 2

NTPDase 2 has a higher ATP/ADP activity ratio (7.2 ± 1.8) in the presence of Ca^{2+} than in the presence of Mg^{2+} (3.5 ± 1.0). Its optimal pH is at pH 5.5-6.5. Under acidic conditions (pH 4), the enzyme has only half activity compared with the optimized condition (Kukulski et al., 2005). Three splice variants: NTPDase 2 α (495 aa), NTPDase 2 β (472 aa), and NTPDase 2 γ (450 aa) were cloned by Matero et al. (2003). When three variants were expressed in Chinese hamster ovary (CHO) cells, only NTPDase 2 α had ATPase activity. Both the NTPDase 2 β and NTPDase 2 γ are missing an important cysteine residue (Cys399) that is responsible for disulfide bond formation (Matero et al., 2003). Mutagenesis of Cys 399 in NTPDase 2 α abolished the enzyme activity, which indicated the importance of its role in protein function. An important physiological function of NTPDase 2 is in the initiation of eye development (Massé et al., 2007).

NTPDase 3

NTPDase 3 has an ATP/ADP activity ratio at 4.3 ± 0.1 in the presence of Ca^{2+} and at 3.5 ± 1.0 in the presence of Mg^{2+} . Their optimized pH condition ranged from pH 7-9 (Kukulski et al., 2005). Two splice forms (NTPDase 3 α and 3 β) were isolated from humans. Although the expressed NTPDase 3 β was plasma membrane localized, it does not perform any enzyme activities due to its lack of the C-terminus ACR5. Co-expression of NTPDase 3 α and 3 β in COS cells altered the amount of active NTPDase 3 α delivered to the plasma membrane which suggested that NTPDase 3 β play roles in regulating the function of NTPDase 3 α (Crawford et al., 2007). Both forms were found abundantly in the neurons of adult brains which overlapped with that of several P2X receptors. This further supporting the important role of ATP mediated signaling in the regulation of sleep and the processing of sensory inputs. (Belcher et al., 2006).

NTPDase 8

NTPDase 8 has an enzyme activity distinct from that of NTPDases 1, 2 and 3. The enzyme had an almost two times higher ATP/ADP activity ratio in the presence of Mg^{2+} (4.1 ± 0.7) than in the presence of Ca^{2+} (2.2 ± 0.1) (Kukulski et al., 2005). It is

more resistant to acidic conditions and maintains enzyme activities from pH 4-8.5. It has been cloned from liver cells (Knowles and Li, 2006).

Extracellular nucleotide signaling and cell surfaced NTPDases in plant cells

A number of studies have demonstrated that plant cells also release significant quantities of ATP into their extracellular matrix (ECM) when they are mechanically stimulated (Jeter et al., 2004), wounded (Song et al., 2006), growing (Kim et al., 2006), or during stomatal opening (Clark et al., 2011). In plants, there may be similar eATP-releasing mechanisms, such as through exocytosis as vesicles fuse with the plasma membrane in active polar growth areas (Clark and Roux, 2011). *M. truncatula* root hairs, when treated with inhibitor of vesicular trafficking brefeldin A will strongly reduce the concentration of eATP, which suggests the importance of secretory vesicle exocytosis for the release of eATP (Kim et al., 2006). In addition, recently a plasma membrane-localized ATP transporter, PM-ANT1, has been postulated to help mediate ATP export during pollen development (Rieder and Neuhaus et al., 2011) (Fig. 1.2).

When applied at sub-micromolar levels, exogenous ATP can induce significant signaling responses in plant cells (Demidchik et al., 2003; Song et al., 2006). In contrast, extensive depletion of eATP can result in loss of cell viability (Chivasa et al., 2005). Thus it has been hypothesized that plant ectoapyrases undertake the important role of helping to control the concentration of eATP. The suppression of AtAPY1 and AtAPY2 activities was proposed to simultaneously increase ATP levels in pollen germination media and inhibit pollen tube growth, and this suppression can also inhibit stomata opening and closure (Wu et al., 2007; Clark et al., 2011).

The plant cell ECM is fairly acidic (pH 5-6) (Felle, 2001). In plants, the ECM of actively growing tissues will be acidified to activate expansin activity. This will increase wall extensibility to enable the cell to take up water and to expand (Rayle and Cleland, 1992). In contrast, the physiological pH of the mammalian cell ECM is between 7 and 8. The enzymes that hydrolyze eATP in mammalian cells have been shown to have their highest activities at neutral pH (Zimmermann et al., 2012). However, compared to the well-characterized biochemical characteristics of mammalian NTPDase, most of the plant

NTPDase studies focused on their physiological roles, which will be discussed in the following section.

Plant cell surface localized NTPDases

Solanum tuberosum

Potato apyrase was long known before it was cloned (Kirshana, 1948). A total of three apyrases (*Stapy1-3*) have been cloned. *Stapy1* was first cloned by Handa and Guidotti (1996). The ATP specific activity of *Stapy1* is about 10,000 mmol Pi/mg/min. Riewe et al. (2008) further cloned *Stapy 2* and *Stapy3* but the *Stapy2* lacks ACR5 and encodes a truncated protein (Riewe et al., 2008). It was long proposed that potato apyrase could be involved in starch synthesis, and Riewe et al. (2008) tested this hypothesis. They found that silencing the potato apyrase gene family with RNA interference (RNAi) decreased potato apyrase activity and led to a general retardation in growth, an increase in the tuber number per plant, and differences in tuber morphology. Microarrays of these apyrase-suppressed mutants showed that transcript levels of cell wall components involved in growth, energy transfer and starch synthesis were increased (Riewe et al., 2008). Riewe et al. (2008) argued for the apoplasmic localization of *Stapy3*-GFP by showing that after its transient expression in leaf epidermis its diffuse signal is quite different from that of the membrane-localized ammonium transporter AtAMT2-GFP (Sohlenkamp et al., 2002).

Medicago truncatula

The *M. truncatula* genome has six apyrase genes. It has been hypothesized that MtAPYRASES (MtAPYs) may be involved in rhizobia symbiosis since *MtAPY1* and *MtAPY4* exhibit an increase in transcript levels upon inoculation of roots with rhizobia (Cohn et al., 2001). However, it has been argued that MtAPYs are regulated by stress and not directly involved in the development of rhizobium symbiosis (Navarro-Gochicoa et al., 2003). No detailed biochemical analysis has been reported for these apyrases.

Dolichos biflorus

The lectin–nucleotide phosphohydrolase (LNP) was isolated from roots of *D. biflorus*. It has four ACRs and can hydrolyze both ATP and ADP, but has no activity with AMP. The K_m of LNP for Mg-ADP is 615 μ M. When LNP binds to Nod factors, its nucleotidase activities are increased. It is localized to the cell membrane, and may play a role in rhizobium–legume symbiosis (Etzler et al., 1999).

Glycine soja

Two apyrases (GS 50 and GS 52) have been isolated from soybean (*Glycine soja*). Biochemical fractionation indicates that GS50 co-localizes with Golgi marker enzymes, while GS52 co-localizes with plasma membrane marker enzymes (Day et al., 2000). RNAi-silenced GS52 in soybean roots would reduce the number of mature nodules. Furthermore, adding extracellular ATP could restore the number of nodules. Taken together these data suggest that the ectoapyrase GS52 is important for nodulation through controlling the eATP concentration (Govindarajulu et al., 2009).

Gossypium hirsutum

Cotton apyrases (GhAPY1 and GhAPY2) were cloned in 2010. Their deduced amino acid sequences are 471 and 469 amino acids in length and share 86% similarities to the well characterized AtAPY1 and AtAPY2. GhAPY1 and GhAPY2 are highly expressed in the elongation stage of the growing fibers. Increased ATP in the ovule culture medium can be detected when the ectoapyrase activity is blocked by the addition of polyclonal anti-apyrase antibodies or apyrase inhibitors. Subsequently, fiber growth is suppressed. This research suggests that GhAPY1 and GhAPY2 function as ectoapyrases that help regulate the [eATP], and that the [eATP], in turn, may regulate cotton fiber elongation (Clark et al., 2010).

Extracellular matrix synthesis and intracellular NTPDases

The extracellular matrix of plant cells is predominantly comprised of three polysaccharides, which are cellulose (40-50%), hemicellulose (20-30%), and pectin (10-20%). These three major building blocks combine with lesser amounts (<10%) of structural glycoproteins (e.g., hydroxyproline-rich extensins), proteoglycans, phenols and

minerals to form the primary cell wall (Chandra et al., 2007; Mckendry, 2002). The most abundant phenolic compound, lignin, can be found together with the polysaccharides in the secondary cell wall (McCann and Roberts, 1991; Henrik and Ulvskov, 2010). Cellulose is a polysaccharide composed of 1,4-linked β -D-glucose residues. Its biosynthesis occurs at the cytosolic side of the plasma membrane by the activity of cellulose synthase proteins (CesA), and then the insoluble cellulose microfibrils are deposited directly into the extracellular matrix.

Hemicelluloses and pectins are made of α -linked or β -linked sugars decorated with side chains that vary in size and composition (Henrik and Ulvskov, 2010; Harholt et al., 2010). In contrast to cellulose, the synthesis of hemicelluloses, pectin and glycoproteins initially occurs in the cytosol and then is finished in the Golgi lumen, whence it is transported to the cell wall via vesicles (Henrik and Ulvskov, 2010; Harholt et al., 2010). The nucleotide sugars (NDP-sugar) utilized in the Golgi lumen for glycan synthesis are transported from the cytosol by the nucleotide sugar transporters in an antiport manner (Reyes and Orellana, 2008). After sugar is added to the targeted moiety, the released NDP is hydrolyzed by the intracellular NTPDase to NMP, which is a substrate for antiport exchange for import of the nucleotide sugars into the Golgi lumen (Liu et al., 2010). Thus, alterations of the NDP or NMP concentration in the Golgi lumen may impair the proper function of glycosyltransferases and the nucleotide sugar transporters.

In mammalian cells, high concentrations of luminal UDP would inhibit glycosyltransferase activity while UMP would not (Khayrad et al., 1974; Kuhn and White, 1977; Brandan and Fleischer, 1982). In contrast, in yeast $\Delta gda1$ mutants, knocking out the GDPases causes the accumulation of GDP in the Golgi lumen and leads to inhibition of the antiport of GDP-mannose. However, the GDP concentrations do not appear to be high enough to inhibit mannosyltransferase (Berninsone et al., 1994). In addition, absence of both NTPDase homologues GDA1 and YND1 in *Saccharomyces cerevisiae* does not totally abolish nucleotide sugar-dependent protein glycosylation (D'Alessio et al., 2005). Studies show that in the absence of NMP, transport can still sustain the entrance of nucleotide sugars until inner and outer concentrations reach

equilibrium, although the efficiency is only one-third that of when preloading occurs with the corresponding NMPs (Puglielli and Hirschberg., 1999; Segawa et al., 2005).

In Arabidopsis, several nucleotide sugar transporters (NSTs) have been functionally characterized: the GDP-mannose transporters GONST1 to GONST5 (Baldwin et al., 2001; Handford et al., 2004); the UDP-galactose/UDP-glucose transporter AtUTr1 (Norambuena, 2002); the UDPgalactose transporters AtUTr2 (Norambuena, 2005), UDPGalT1 and UDPGalT2 (Bakker et al., 2005) and AtNST-KT (Rollwitz et al., 2006). The antiport mode has only been tested in the AtNST-KT, where the entrance of UDP-galactose is dependent on the exit of UMP (Rollwitz et al., 2006). Up until now, there is no information about how Arabidopsis intracellular NTPDases work together with the NSTs or the glycosyltransferase to regulate the nucleotide sugar entry to the Golgi and regulate the glycan synthesis.

Intracellular localized NTPDase

Intracellular NTPDases are found in the Golgi (NTPDase 4, 6, YND1), ER (NTPDase 5) and intracellular vesicles (NTPDase 7) and undertake functions distinct from the cell surface NTPDases (Knowles, 2011). The protein structures are more diverse than the surface-localized NTPDases. They may have TMDs at both their N- and C-termini (NTPDase 4 and 7) or only one TMD on either the N- (NTPDase 5 and 6) or C-terminus (YND1), and have only four to six cysteine residues.

NTPDase 4

NTPDase 4 was first cloned by Wang and Guidotti (1998) from a human brain cDNA library. It can hydrolyze GDP, CDP, TDP at comparable rates, with UDP as the best substrate. However, its ADPase activity was negligible. Its UDPase activity is higher in the presence of Ca^{2+} than in Mg^{2+} . Its activity towards NTPs is more than 30% less than that toward NDPs. Immunofluorescence staining showed that NTPDase 4 was localized in the Golgi apparatus. Further biochemical analysis showed that its active site is on the luminal side of Golgi, not extracellular (Wang and Guidotti; 1998).

NTPDase 5

The human NTPDase 5 was cloned from a macrophage cDNA library. It will be secreted after cleavage of its signal peptide (amino acids 1-20). The processed NTPDase5 should be around 45.5 kDa. However, the band detected from media extract is slightly larger than in the crude extract, which is about 50 kDa. Mulero et al. (1999) explained this 5 kDa difference as being due to glycosylation. NTPDase 5 prefers to hydrolyze NDPs over NTPs. The best substrate for NTPDase 5 is UDP. Its UDPase activity is almost four times higher than its ADPase activity (Mulero et al., 1999). Another soluble form of NTPDase 5 has been isolated from bovine liver. This form of the enzyme again shows preference to hydrolyze UDP, GDP and CDP but not ADP. Immunolocalization studies indicate it has primarily an ER distribution (Trombetta and Helenius, 1999).

NTPDase 6/GDA1

NTPDase 6 is characterized as a GDPase due to its dominant GDPase activity over other nucleotide substrates. The human NTPDase 6 was cloned by two groups (Yeung et al., 1999 and Hicks-Berger et al., 2000). It has both membrane-bound and soluble forms. Its highest hydrolysis activity is with GDP and IDP substrates. Its UDPase activity is about one third of its GDPase activity, and it has negligible ADPase activity (Hicks-Berger et al., 2000). Its GDPase activity does not change whether the enzyme has or does not have an N-link glycan. This result falsifies the hypothesis that the deglycosylation of eNTPDase may decrease its activity, which was observed in NTPDase 1 and 3 (Hicks-Berger et al., 2000). Its immunofluorescence signal can be detected most abundantly in the Golgi, but some cell-surface localization can also be observed (Braun et al., 2000).

GDA1 from *Saccharomyces cerevisiae* was the first NTPDase to be cloned (Abeijon et al., 1993). Based on biochemical analyses, it is mainly a GDPase and has little UDPase activity. It does not hydrolyze other nucleotides (Plesner, 1995). The physiological significance of GDA1 is that it locates in the Golgi lumen to hydrolyze GDP to GMP for the exchange of the nucleotide sugar from the cytosol. Deletion of GDA1 results in a marked reduction in Golgi mannosylation of proteins and lipids *in vivo* and causes a 4-fold lower rate of GDP-mannose entry into Golgi vesicles in dolichol-phosphate- mannose synthase-deficient cells (Abeijon et al., 1993; Berninsone et al.,

1994). A similar enzyme was also cloned from fission yeast *Schizosaccharomyces pombe* (SPgda1), but it hydrolyzes both UDP and GDP (D' Alession et al., 2003).

YND1

The *YND1* gene is a homologue of *GDA1* in *Saccharomyces cerevisiae*. The protein structure of YND1 has one C-terminal TMD, which distinguishes it from the other NTPDase members. Recently it was suggested that YND1 should be categorized as NTPDase 9 (Knowles, 2011). YND1 has much broader substrate specificity and can hydrolyze both nucleoside tri- and diphosphates, but GDP is still its best substrate. It is worth to note that among all the intracellular NTPDases, YND1 is the only one with noticeable ADPase activity. Its function overlaps that of GDA. Knocking out both proteins in *S. cerevisiae* causes the loss of *N*- and *O*- linked glycosylation and impairs cell-wall formation and cell growth (Gao et al., 1999). However, the disruption of both genes in *Schizosaccharomyces pombe* was lethal (D' Alession et al., 2003).

NTPDase 7

NTPDase 7 (LALP1) was cloned from both human and mouse brains. An LALP1-GFP fusion protein had an intracellular vesicle distribution. The enzyme can hydrolyze NTPs as well as NDPs, and has higher activity toward UTP than UDP (Shi et al., 2001). No physiological significance has been reported yet.

Plant intracellular NTPDase

***Oryza sativa* L. cv Nipponkai**

An NTPDase has been purified from Triton X-100 solubilized Golgi membranes of rice (*Oryza sativa* L. cv Nipponkai) suspension cells. The optimal pH for its enzyme activity was around 7. It can hydrolyze IDP, UDP, and GDP effectively in the presence of Mg^{2+} . However, its activity with ADP, CDP was only 10 to 20% of IDP (Mitsui et al., 1994).

Pisum sativum

The psNTP9 was originally purified from nuclei of etiolated pea plumules, where it may be regulated by light, calmodulin and casein kinase II (Chen and Roux 1986, Chen et al., 1987; Hsieh et al., 1996; Hsieh et al., 2000). However, it may have a dual localization, because it could also be found associated with purified plasma membranes and with fractions purified from the extracellular matrix (Thomas et al., 1999). A functional analysis showed that when expressed in *Arabidopsis* psNTP9 could improve phosphate uptake from the extracellular space, and, when expressed in yeast, it could restore growth on low Pi in a Pi-uptake deficient yeast mutant (*Pho84*; Thomas et al., 1999). Also, its constitutive expression in *Arabidopsis* could confer xenobiotic resistance, in part due to its ectophosphatase activity (Thomas et al., 2000).

Other studies have demonstrated the existence of a pea apyrase involved in cytoskeleton-related signal transduction and transportation (Shibata et al. 1999), and a pea Golgi NDPase that uses UDP as a substrate and performs similar functions to those outlined in *S. cerevisiae* (Orellana et al., 1997; Neckelmann and Orellana, 1998).

APYRASEs in Arabidopsis

In *Arabidopsis* a total of seven apyrases have been identified. Currently, only APYRASE 1 (AtAPY1: At3g04080) and APYRASE 2 (AtAPY2: At5g18280) have been characterized. These two apyrases share 87% identity at the amino acid level, and both contain the typical five ACRs, an ATP-binding motif, and a hydrophobic segment at the N-terminus. A calmodulin (CaM)-binding domain is found only in AtAPY1. Bacterially expressed AtAPY1 and AtAPY2 display enzymatic properties characteristic of apyrases, such as the hydrolysis of ATP and ADP but not AMP while both are insensitive to inhibitors of ATPases (Steinebrunner et al., 2000).

AtAPY1 and AtAPY2 play a key role in pollen germination and vegetative growth. The single knock-out mutants do not show any discernible phenotype due to the functional redundancy of these two apyrases. However, in the *apy1 apy2 double knockout* (*apy1 apy2 dKO*) pollen failed to germinate, which implicated this step as the cause of the sterility of apyrase DKO mutants. Pollen grains were similar to wild type in size, shape, and nuclear state and were viable as assayed by metabolic activity and plasma membrane integrity when observed under light microscopy (Steinebrunner et al., 2003).

Due to the sterility of *apy1 apy2* dKO plants, RNA interference (RNAi) was performed as an alternative approach to silence the expression of apyrases. The RNAi lines thus silenced display a dwarf phenotype in overall vegetative growth and have dramatically reduced growth in primary roots and etiolated hypocotyls (Wu et al., 2007).

These previous results imply that in plants AtAPY1 and AtAPY2 may act like their animal orthologues to regulate the concentration of extracellular nucleotides, and lead to changes of growth in *Arabidopsis*. However, only antibody inhibition data in pollen suggest they may have ectophosphatase functions related to the phenotypes observed (Wu et al., 2007). Moreover, proteomic study has demonstrated that AtAPY2 may associate with Golgi apparatus (Dunkley et al. 2004).

Five more apyrases (AtAPY3-7) have been cloned from *Arabidopsis*. They also contain five ACRs. Based on the q-PCR analysis, AtAPY6 is abundantly expressed in pollen. In contrast, AtAPY7 is expressed ubiquitously in various tissues. Knocking out *AtAPY6* and *AtAPY7* caused late anther dehiscence and low male fertility. The *AtAPY6* tagged YFP shows intracellular punctuate structures. Knocking out *AtAPY 3, 4, 5* simultaneously by RNAi in either wildtype or *apy6* background did not show obvious altered phenotypes (Jian Yang, Ph. D. dissertation, 2011).

Summary

In this dissertation, I tested the possible localization and biochemical function of all the *Arabidopsis* apyrases members. Chapter 2 focuses on the physiologically well-characterized AtAPY1 and AtAPY2. I demonstrated that the stable-transformation of sterile double knockout plants (*apy1apy2* dKO) with AtAPY1-GFP and AtAPY2-GFP can rescue the lethal dKO mutants, which suggested the fusion proteins have proper function and localization. Using this approach I discovered that AtAPY1 and AtAPY2 localize in the Golgi. I further tested the possibilities of their function in the Golgi by complementing the N-link glycosylation defect in yeast $\Delta gda1$ mutants. Microsomes from wild-type plants showed robust UDPase/GDPase activity. Knocking out AtAPY1 or AtAPY2 could reduce UDPase, but only knocking out APY2 could significantly reduce the GDPase activity. Furthermore, I also examined the possibility that AtAPY1 and AtAPY2 regulated UDP galactose transport and assayed the effects of their knockouts on

the composition of the cell wall. The UDP galactose transport was not altered in either knockout mutant, nor did either mutant show any significant alteration of the cell wall composition. Chapter 3 expands the focus to the other members (AtAPY3-7). The transiently expressed AtAPY3-7-YFP showed endomembrane associations. Only APY3-5 can be successfully expressed in the yeast, and each of these apyrases showed different levels of complementation of N-link glycosylations in yeast *gda* mutants. Heterologous expression of none of the APY3-5 can rescue the hygromycin sensitivity of yeast *ynd* mutants.

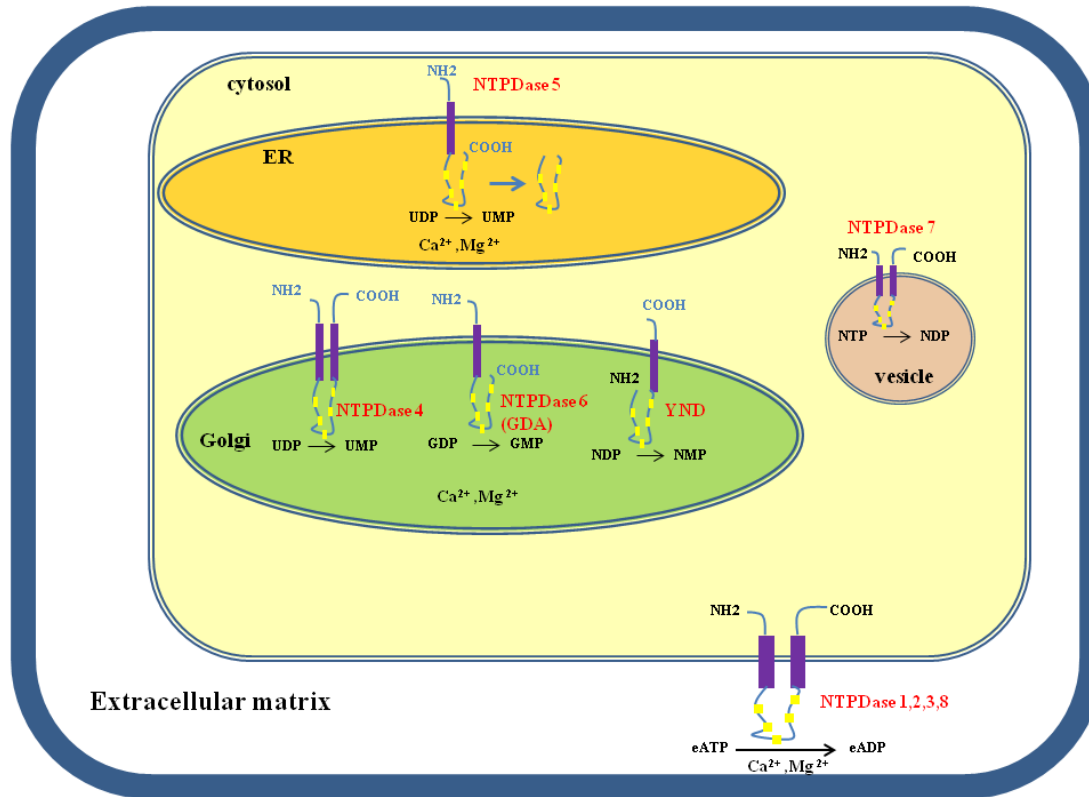


Figure. 1.1. The GDA1_CD39 superfamily. The NTPDase 1, 2, 3 and 8 localize on the plasma membrane and are involved in the hydrolysis of extracellular ATP. The NTPDase 4, 5, 6 and YND are ER/Golgi-resident proteins. They hydrolyze the nucleotide diphosphates that are derived from the nucleotide sugars. The NTPDase 7 is an NTPDase localized in as yet undefined intracellular vesicles.

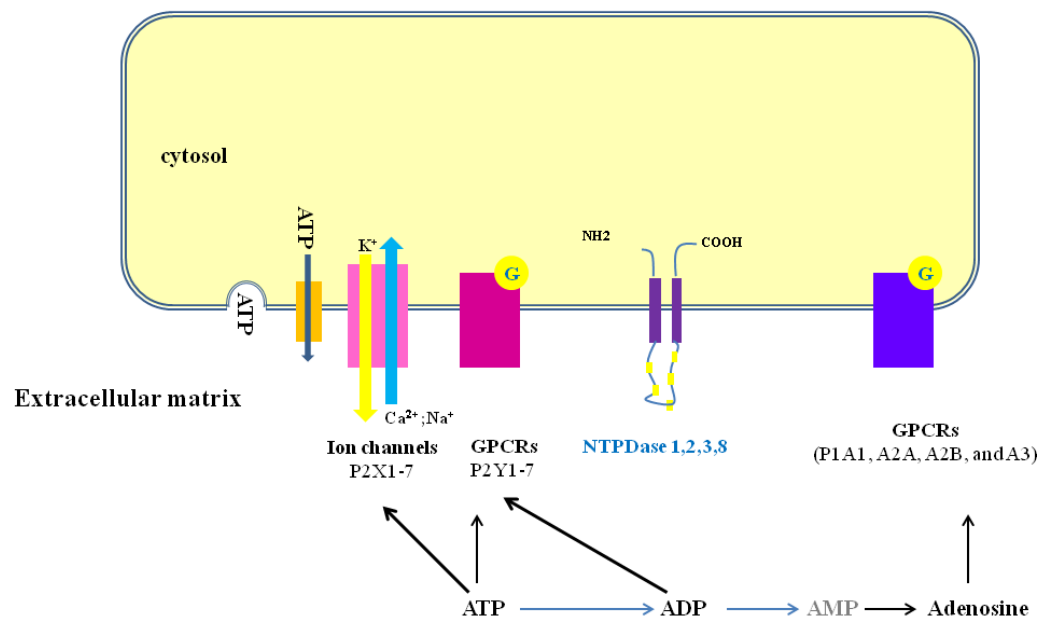


Figure 1.2. Model of how ATP serves as a signaling molecule in the extracellular matrix (ECM) in mammalian cells (modified from Zebisch & Sträter, 2007). The ATP can be delivered to the ECM through secretory vesicles or pumped out by transporters. Plasma membrane-associated NTPDases hydrolyze ATP to ADP and further hydrolyze ADP to AMP to prevent the desensitization of the receptors (P2X, P2Y). The AMP can be metabolized by other enzymes to adenosine, which is the ligand for P1 receptors.

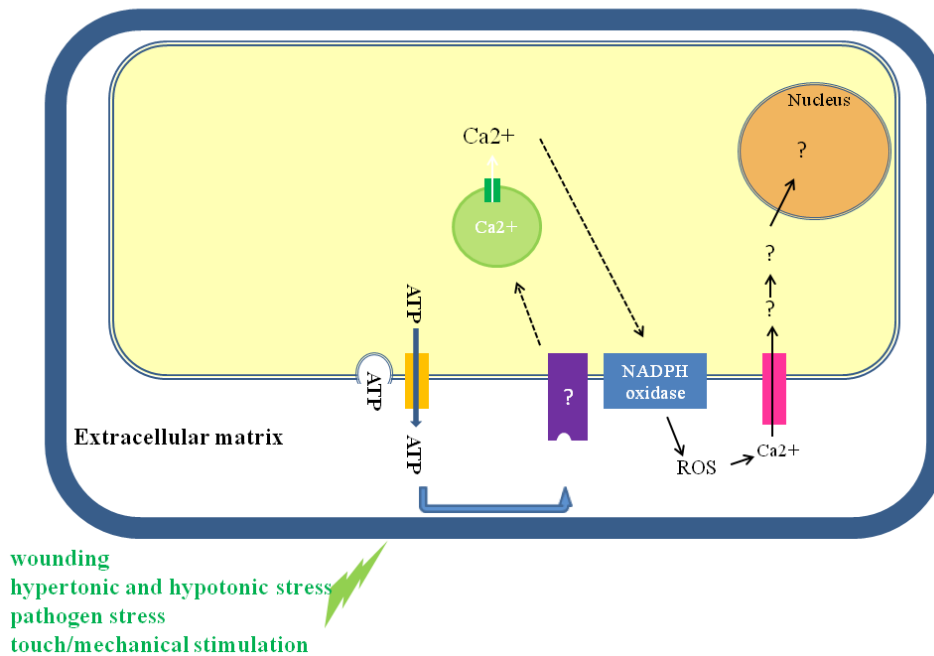


Figure 1.3. A model of how ATP may serve as a signaling molecule in the extracellular matrix (ECM) in plant cells (modified from Clark and Roux, 2011). The ATP can be delivered to the ECM through secretory vesicles or pumped out by transporters (PM-ANT1). The extracellular ATP may bind to currently unknown receptor(s) and trigger the release of internally stored Ca^{2+} and further induce ROS production. The ROS can open plasma membrane-localized Ca^{2+} channels; and further increase the cytosolic Ca^{2+} concentration.

Chapter 2. AtAPY1 and AtAPY2 Function as Golgi localized Nucleoside Diphosphatases in *Arabidopsis thaliana*

Abstract

NTPDases (Apyrases) (EC 3.6.1.5) hydrolyze di- and triphosphate nucleotides, but not monophosphate nucleotides. They are categorized as E-type ATPases, have a broad divalent cation (Mg^{2+} , Ca^{2+}) requirement for activation, and are insensitive to inhibitors of F-type, P-type, and V-type ATPases. Among the seven NTPDases identified in *Arabidopsis*, only APYRASE 1 (AtAPY1) and APYRASE 2 (AtAPY2) have been previously characterized. In this work, either AtAPY1 or AtAPY2 tagged with C-terminal green fluorescence protein (GFP) driven by their respective native promoter can rescue the *apy1 apy2* double knockout (*apy1 apy2 dKO*) successfully, and confocal microscopy reveals that these two *Arabidopsis* apyrases reside in the Golgi apparatus. In *Saccharomyces cerevisiae*, both AtAPY1 and AtAPY2 can complement the Golgi-localized GDA1 mutant rescuing its aberrant protein glycosylation phenotype. In *Arabidopsis*, microsomes of wildtype show higher substrate preferences toward UDP compared to other NDP substrates. Loss-of-function *Arabidopsis* AtAPY1 mutants exhibit reduced microsomal UDPase activity, and this activity is even more significantly reduced in the loss-of-function AtAPY2 mutant and in the AtAPY1/AtAPY2 RNAi technology repressor lines. Microsomes from wildtype plants also have detectable GDPase activity, which is significantly reduced in *apy2* but not *apy1* mutants. The GFP tagged AtAPY1 or AtAPY2 constructs in the *apy1 apy2 dKO* plants can restore microsomal UDP/GDPase activity confirming that they both also have functional competency. The cell walls of *apy1*, *apy2* and the RNAi silenced lines all have increased composition of galactose, but the transport efficiency of UDP-galactose across microsomal membranes was not altered. Taken together these results reveal that AtAPY1 and AtAPY2 are Golgi localized nucleotide diphosphatases and are likely to have roles in regulating UDP/GDP concentrations in the Golgi lumen.

Key words: *Arabidopsis*, Golgi NTPDase, Glycosylation, functional complementation

Abbreviations: AtAPY1 and AtAPY2, Arabidopsis APYRASE 1 and 2; GFP, Green Fluorescence protein; *dKO*, double knockout; WT, wildtype

Introduction

The nucleoside triphosphate diphosphohydrolases (NTPDase; NDPase) (EC 3.6.1.5) or apyrases are currently grouped within the GDA1_CD39 superfamily. The members of this enzyme type contain five apyrase conserved regions (ACRs) and are widely identified in eukaryotic cells (Knowles, 2011). They are enzymes that hydrolyze both di- and triphosphate nucleotides, with biochemical characteristics of broad divalent cation (Mg^{2+} , Ca^{2+}) requirements for activation, and insensitivity to inhibitors of F-type, P-type, and V-type ATPases (Zimmermann, 2001). NTPDases can be found on the cell surface (NTPDase type 1 to 3 and 8) or intracellularly (NTPDase type 4 to 7 and YND1) and undertake various physiological functions within the cell (Knowles, 2011).

In animal cells, the best known cell surface localized NTPDase is a member of the type 1 class and regulates platelet aggregation by terminating signal transduction initiated by nucleotides such as extracellular ATP (eATP) (Gayle et al., 1999; Buerger et al., 2005). Cell surface NTPDases are also involved in initiating eye development, neural development and differentiation (NTPDase 2); modulate behavior (NTPDase 3) and have unknown physiological functions (NTPDase 8) (Knowles, 2011). In plants, apoplastic apyrases have been observed in *Medicago truncatula* and potato (*Solanum tuberosum*). Their functions are diverse and are involved in rhizobia symbiosis, plant growth and development. The *M. truncatula* genome has six apyrase genes. It was hypothesized that MtAPYRASES (MtAPYs) may be involved in rhizobia symbiosis since MtAPY1 and MtAPY4 exhibit an increase in transcript levels upon inoculation of roots with rhizobia (Cohn et al., 2001). However, it has been argued that MtAPYs are regulated by stress and not directly involved in the development of rhizobium symbiosis (Navarro-Gochicoa et al., 2003). In potato, a total of three apyrases have been identified. Silencing of the potato apyrase gene family with RNA interference (RNAi) constructs led to a general retardation in growth, an increase in the tuber number per plant, and differences in tuber morphology. Transcript levels of cell wall components involved in growth, energy transfer and starch synthesis were increased in microarrays of these apyrase suppressed mutants (Riewe et al. 2008).

A number of studies have demonstrated that plant cells also release significant quantities of ATP into their extracellular matrix when they are mechanically stimulated

(Jeter et al., 2004), wounded (Song et al., 2006), growing (Kim et al., 2006) or during stomatal opening (Clark et al., 2011). It has also been reported that plant cells exhibit significant signaling responses to the exogenous application of sub-micromolar ATP (Demidchik et al., 2003; Song et al., 2006) and that extensive depletion of eATP can result in loss of cell viability (Chivasa et al., 2005). Thus, in order to control the concentration of eATP, it has been hypothesized that plant ecto-apyrases undertake this role. The suppression of ecto-apyrase activities by anti-apyrase antibodies can simultaneously increase ATP levels in pollen germination media and inhibit pollen tube growth, and it can also inhibit stomata opening and closure (Wu et al., 2007; Clark et al., 2011).

Intracellular NTPDases are found in the Golgi (NTPDase 4, 6, YND1), ER (NTPDase 5) and intracellular vesicles (NTPDase 7) and undertake functions distinct from the cell surface NTPDases (Knowles, 2011). The most characterized are the yeast GDA1/YND1 enzymes, which are involved in maintaining protein glycosylation in the Golgi apparatus (Abeijon et al. 1992, Gao et al., 1999). Nucleotide sugars are synthesized in the cytosol and transported into the Golgi lumen as the substrate donor for the glycosyltransferase reactions (Abeijon et al., 1992; Capasso and Hirschberg, 1984). Following glycosylation of luminal substrates such as protein and lipid acceptors, released nucleoside diphosphates (NDPs) are converted to nucleoside monophosphate (NMPs) by NTPDases. It is proposed that NMP may exit the lumen in a coupled, equimolar exchange, with cytosolic nucleotide sugars. In the absence of NTPDases the subsequent decrease in available NMPs for antiport should lead to reduced entry of nucleotide sugars into the Golgi lumen, and thus impaired sugar chain synthesis in the Golgi (Liu et al., 2010).

The *S. cerevisiae* GDA1 enzyme (NTPDase 6) is a Golgi luminal guanosine diphosphatase (GDPase). Based on the biochemical analysis, it can serve both as a GDPase and UDPase with preferential activity towards the hydrolysis of GDP over UDP. It does not hydrolyze other nucleotides (Plesner, 1995). Deletion of GDA1 results in a marked reduction in Golgi mannosylation of proteins and lipids *in vivo* and causes a 4-fold lower rate of GDP-mannose entry into Golgi vesicles in dolichol-phosphate-mannose synthase-deficient cells (Abeijon et al., 1993; Berninsone et al., 1994). The *S.*

cerevisiae *YND1* gene is a homologue of *GDA1* but has broad substrate specificity and can hydrolyze nucleoside tri- and diphosphates. The functions of *GDA1* and *YND1* partially overlap. *S. cerevisiae* lacking both genes lose *N*- and *O*- linked glycosylation and have defects in cell-wall formation and cell growth (Gao et al., 1999). The *YND1* enzyme has only one C-terminal transmembrane domain in contrast to other NTPDases, which contain either two transmembrane domains or one transmembrane domain at the N-terminal. Recently it was suggested that *YND1* should be categorized as NTPDase 9 (Knowles, 2011).

The intracellular classes of NTPDases are also found in plants. In pea, the apyrase psNTP9 was originally purified from nuclei of etiolated pea plumules, where it may be regulated by light, calmodulin and casein kinase II (Chen and Roux 1986, Chen et al., 1987; Hsieh et al., 1996; Hsieh et al., 2000). However, it may have a dual localization, because it could also be found associated with purified plasma membranes and with fractions purified from the extracellular matrix (Thomas et al., 1999). A functional analysis showed that when expressed in *Arabidopsis* psNTP9 could improve phosphate uptake from the extracellular space, and, when expressed in yeast, it could restore growth on low Pi in a Pi-uptake deficient yeast mutant (*Pho84*; Thomas et al., 1999). Also, its constitutive expression in *Arabidopsis* could confer xenobiotic resistance, in part due to its ectophosphatase activity (Thomas et al. 2000). Other studies have demonstrated the existence of a pea apyrase involved in cytoskeleton-related signal transduction and transportation (Shibata et al. 1999), and a pea Golgi NDPase that uses UDP as a substrate and performs similar functions to those outlined in *S. cerevisiae* (Orellana et al., 1997; Neckelmann and Orellana, 1998).

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et al., 2000). AtAPY1 and AtAPY2 play a key role in pollen germination and vegetative growth. The single knock-out mutants do not show any discernible phenotype due to the functional redundancy of these two *Arabidopsis* apyrases. However, in the *Arabidopsis atapy1 atapy2 double knockout (apy1 apy2 dKO)* pollen failed to germinate, which implicated this step as the cause of the sterility of *apy1 apy2 dKO* mutants. Pollen grains were similar to wildtype in size, shape, and nuclear state and were viable as assayed by metabolic activity and plasma membrane integrity when observed under light microscopy (Steinebrunner et al., 2003). Due to the sterility of *apy1 apy2 dKO*, RNA interference (RNAi) was performed as an alternative approach to silence the expression of *Arabidopsis* apyrases. The RNAi lines thus silenced display a dwarf phenotype in overall vegetative growth and have dramatically reduced growth in primary root and etiolated hypocotyls (Wu et al., 2007).

These previous results imply that in plants AtAPY1 and AtAPY2 may act like their animal orthologues to regulate the concentration of extracellular nucleotides, and lead to changes of growth in *Arabidopsis*. As noted previously (Wu et al., 2007; Clark et al., 2011), because secretory vesicles are thought to be a key source of extracellular nucleotides (Geigenberger et al., 2009), AtAPY1 and AtAPY2 could control the concentration of extracellular nucleotides by their activity in the Golgi lumen and/or by their activity on the plasma membrane. However, only antibody inhibition data in pollen suggest they may have ectophosphatase functions related to the phenotypes observed (Wu et al. 2007). Here we generated the green fluorescence tagged AtAPY1 and AtAPY2 and demonstrate that they are Golgi localized and provide evidence to support their role as intracellular NTPDases.

Results

Both AtAPY1-GFP and AtAPY2-GFP can complement *apy1 apy2* dKO plants

Since NTPDases have been reported to have various localizations within the cell and play a variety of functional roles at these different locales, we investigated subcellular localization of AtAPY1 and AtAPY2, using genomic clones with C-terminal GFP fusions. Previous reports had shown that pollen lacking both AtAPY1/AtAPY2 is infertile, thus *apy1 apy2* dKO cannot be obtained (Steinebrunner et al., 2003). C-terminal GFP fusion constructs comprising either AtAPY1 or AtAPY2 were stably transformed into either *APY1apy1 apy2apy2* or *apy1apy1 APY2apy2* plants. The rescued transformants were screened using 3'-UTR specific primer sets that can distinguish native AtAPY1 and AtAPY2 from the *APY-GFP* inserts (Fig. 2.1A and B and Fig. 2.7). These T2 lines were initially screened for the presence of T-DNA insertions in both the AtAPY1 and AtAPY2 loci (data not shown). Thus, segregation of *PROAPY1:APY1-GFP* (or *PROAPY2:APY2-GFP*) transformants should follow a Mendelian inheritance and display a 2:1 segregation ratio between AtAPY1 or AtAPY2 and T-DNA inserts (*apy1* or *apy2*) in the T2 generation (Fig. 2.1A and B). Native AtAPY1 or AtAPY2 mRNA could not be detected by RT-PCR in the *apy1 apy2* dKO rescue lines containing the *APY-GFP* constructs (Fig. 2.8). No segregation of T-DNA alleles occurred in the T3 generations, providing further genetic evidence that the *PROAPY1:AtAPY1-GFP* or *PROAPY2:AtAPY2-GFP* fusion constructs can rescue the *apy1 apy2* dKO plants through proper function and correct localization (Fig. 2.1C). The *apy1 apy2* dKO seedlings expressing either *PROAPY1:APY1-GFP* or *PROAPY2:APY2-GFP* show no discernible phenotype, which is in agreement with the absence of visual phenotype in the single *apy1* or *apy2* mutants and also supports the notion that AtAPY1 and AtAPY2 play redundant functions in Arabidopsis (Fig. 2.1D). Attempts to fuse GFP to the N-terminal of AtAPY1 and AtAPY2 failed to complement *apy1 apy2* dKO lines suggesting the importance of the N-terminal region for proper function/localization (data not shown).

AtAPY1 and AtAPY2 functionally localize to the Golgi apparatus

The *apy1 apy2 dKO* plants containing either the *PROAPY1:APY1-GFP* or the *PROAPY2:APY2-GFP* showed similar punctate structures (Fig. 2.2). The cis-Golgi marker Man49-mCherry (Fig. 2.2A and C) and the plasma membrane-specific marker aquaporin PIP2A-mCherry (Fig. 2.2B and D) were each stably transformed into both the *PROAPY:APY-GFP* rescue lines to aid visualization and confirm the identity of these punctate structures (Saint-Jore-Dupas et al., 2006; Nelson et al., 2007). The signal from both AtAPY1-GFP and AtAPY2-GFP in the *apy1 apy2 dKO* background co-localized with the cis-Golgi marker, indicating their functional residency in the Golgi apparatus (Fig. 2.2A and C). Furthermore, we also examined the AtAPY1-GFP and AtAPY2-GFP signal in pollen tubes where AtAPY1 and AtAPY2 are abundantly expressed (Fig. 2.2E). The internal punctate signal is also observed. Unfortunately, the cis-Golgi marker Man49-mCherry and the plasma membrane-specific marker aquaporin PIP2A-mCherry are driven by 35S promoter, which is only weakly expressed in the pollen tubes (Wilkinson et al., 1997). Several pollen tubes were examined but no marker signals could be obtained to demonstrate marker co-localization. Confocal images obtained from both root and pollen tubes consistently showed internal punctuate structures and no signal could be discerned to support the localization of AtAPY1 or AtAPY2 to the plasma membrane (Fig. 2.2B and D).

Immunoblot analysis of complemented *apy1 apy2 dKO* lines with antibodies against GFP was undertaken to ensure the integrity of the AtAPY1-GFP and AtAPY2-GFP constructs in these lines and to confirm the Golgi-based GFP signal corresponded to the intact protein constructs (Fig. 2.3 and Fig. 2.9). Both total protein and microsomal fractions from complemented plants were examined. Analysis of complemented lines resulted in a GFP specific signal that coincided with the predicted molecular weight of the heterologous proteins, AtAPY1-GFP (predicted 78 kDa) and AtAPY2-GFP (predicted 78 kDa) (<http://www.scripps.edu/~cdputnam/protcalc.html>). The analysis also indicated that the AtAPY1 appears to undergo N-terminal processing (approximately 3 kDa) after localization to the microsomal fraction (Fig. 2.3).

AtAPY1 and AtAPY2 are functionally similar to GDA1 of *Saccharomyces cerevisiae*

The yeast apyrases GDA1 and YND1 are functionally characterized Golgi resident NDPases which exhibit protein glycosylation and cell wall phenotypes when disrupted. In order to investigate the functional roles of the AtAPY1 and AtAPY2 proteins in the Golgi apparatus we undertook a complementation analysis of the yeast $\Delta gda1$ and $\Delta ynd1$ mutants. Constructs containing either AtAPY1 or AtAPY2 were transformed into both $\Delta gda1$ and $\Delta ynd1$ yeast cells.

The vacuolar enzyme carboxypeptidase Y (CPY) with four N-linked carbohydrate chains is an excellent marker enzyme to assess *N*-glycosylation in yeast (Stevens et al., 1982). As previously demonstrated, CPY migrates faster in the $\Delta gda1$ background due to changes in *N*-glycosylation when compared to WT yeast (Fig. 2.4A). The expression of either AtAPY1 or AtAPY2 in the $\Delta gda1$ background could successfully revert this increased mobility suggesting that the Golgi localized AtAPY1 and AtAPY2 can functionally replace the yeast GDA1 protein (Fig. 2.4A). A similar finding with only AtAPY1 was also recently observed using an identical approach (Parsons et al., 2012).

Interestingly, no mobility changes of CPY were observed in the $\Delta ynd1$ yeast cells lacking the second yeast apyrase YND1 (a homologue of GDA1). To our knowledge this finding has not been previously reported, even though this mutant is known to display a glycosylation phenotype (Gao et al., 1999). Thus, in order to determine whether either AtAPY1 or AtAPY2 were also able to replace YND1 function, we investigated their ability to rescue another characterized phenotype exhibited by $\Delta ynd1$ cells. The $\Delta ynd1$ mutation results in an increased sensitivity to the aminoglycoside hygromycin B due to reduced protein glycosylation or defective cell wall biosynthesis (Gao et al., 1999). The sensitivity assay indicates that while $\Delta ynd1$ cells are indeed sensitive to hygromycin B; neither the AtAPY1 nor the AtAPY2 gene products could restore hygromycin B tolerance (Fig. 2.4B). Taken together, these results provide evidence that the Golgi-localized AtAPY1 and AtAPY2 are capable of functionally complementing the Golgi-localized yeast apyrase GDA1 but not the functionally similar yeast apyrase YND1.

NDPase activity is reduced in microsomes of Arabidopsis apyrase mutants

In an attempt to determine whether AtAPY1 and AtAPY2 function in the plant Golgi apparatus, we examined NDPase activity in the Arabidopsis apyrase mutant lines using various NDP substrates (ADP, UDP, CDP or GDP). Microsomes were isolated from 7-day old Arabidopsis seedlings and NDPase activity measured using the malachite green assay. Microsomal preparations of wildtype Arabidopsis (WS) show higher enzyme activity towards UDP when compared to the other NDP substrates (Fig. 2.5). The loss of either AtAPY1 or AtAPY2 resulted in a significant reduction in UDPase activity in microsomal membrane preparations. The loss of AtAPY2 seemed to have a greater impact on UDPase activity in comparison to the loss of AtAPY1 and it was equivalent to that measured in the RNAi suppressor line R2-4A (*APY1-RNAi apy2*). A lack of AtAPY1 in the single mutant did not significantly reduce GDPase activity compared to microsomes isolated from wildtype, supporting the notion that AtAPY2 may have a substrate preference for GDP when compared to AtAPY1. The NDPase activities measured in the RNAi suppressor line R2-4A are similar to those measured in the *apy2* mutant. This result likely reflects the fact that the RNAi only reduces AtAPY1 in the *apy2* background and thus residual AtAPY1 protein may be present in microsomes from 7-day old plants. Both the AtAPY1-GFP and AtAPY2-GFP lines can restore the UDPase/GDPase activities in the *apy1 apy2 dKO* mutant lines, which biochemically confirm the function of the fusion protein (Fig. 2.5). There was no measured difference in microsomal ADPase activity among the mutants examined (Fig. 2.5). Microsomes isolated from *apy2* plants and the *APY2-GFP* line2 were also significantly reduced in CDPase activity. However, this has little physiological significance since as far as we are aware there is no CDP substrate in the Golgi lumen. Taken together these data indicate that both AtAPY1 and AtAPY2 function as UDPase/GDPase.

Matrix polysaccharide composition and UDP-galactose transport in the Arabidopsis apyrase mutants

Given the complementation in yeast and reduction of UDPase/GDPase activities in APY mutants, it has been proposed that a reduction in NDP hydrolysis and the reduction of substrates for the nucleotide sugar exchange between the Golgi lumen and the cytosol could result in reduced glycosyltransferase activity and lead to cell wall defects (D'Alessio et al., 2005). We evaluated the monosaccharide composition from cell wall extracts from the three apyrase mutant lines (Table 2.1). Overall, monosaccharide content from cell wall extracts of the mutant lines was not altered with the exception of galactose, which was significantly higher in all Arabidopsis apyrase mutant backgrounds (Fig. 2.6A). Consequently, we sought to investigate the UDP-galactose transport efficiency in Golgi-enriched membrane fractions of the RNAi suppression line R2-4A. This line exhibits a drastic growth phenotype due to the inhibition of both AtAPY1 and AtAPY2. Unexpectedly, UDP-galactose transporter rates in microsomes isolated from 2-week-old seedlings of the R2-4A line revealed that UDP-galactose transport was similar to that measured in the wildtype (Fig. 2.6B).

Discussion

Glycan synthesis is important for proper formation of the extracellular matrix of cells, and for protein/lipid glycosylation. The process thus plays a critical role in supporting normal cell growth and organ development in diverse organisms. The Golgi resident nucleoside diphosphatase has been long known to help catalyze steps in this process (Seifert, 2004). To date little detail has been reported concerning these Golgi nucleoside diphosphatases. In this study, we show that Golgi-localized AtAPY1 and AtAPY2 function as nucleoside diphosphatases based on subcellular localization evidence from fluorescence tags, biochemical analysis in Arabidopsis and complementation assays in yeast.

Subcellular localization

The pollen that carries *apy1 apy2* alleles cannot germinate and results in the inability to generate double mutants (Steinebrunner et al., 2003). Conditional double knockout seedlings can be generated by complementing the *apy1 apy2 dKO* mutants with AtAPY2 genes driven by a pollen specific promoter. However, these plants do not grow or develop past the seedling stage (Wolf et al., 2007). The C-terminal fluorescent protein tagged AtAPY1 and AtAPY2 rescued the *apy1 apy2 dKO* mutant phenotype indicating both their functional and spatial viability (Fig. 2.1). The most abundant subcellular signals were observed in punctate structures that were confirmed as Golgi structures when transformed with the cis-Golgi marker (Fig 2.2A and C). This result is in agreement with previously published Golgi proteomic data indicating AtAPY2 is in the Golgi (Dunkley et al., 2004), and a more recent Arabidopsis Golgi proteomic survey identifying both AtAPY1 and AtAPY2 and confirming localization by transient fluorescent markers (Parsons et al., 2012). Recently, Schiller et al. (2012) reported that a 35S driven AtAPY1-GFP construct also displays Golgi localization. Our work also confirmed these findings by demonstrating the Golgi localization of both AtAPY1 and AtAPY2 through rescue of the *apy1 apy2 dKO* mutant line by its native promoter. Taken together these data present an extremely strong argument for the Golgi-based subcellular location of AtAPY1 and AtAPY2, and for their role as NTPDases.

Based on von Heijne predictions, the N-terminal transmembrane domain of AtAPY1 and AtAPY2 may have a putative cleavage site after residue 35 (Fig 2.10) (Steinebrunner et al., 2000). Schiller et al. (2012) have reported that AtAPY1 is an integral membrane protein. However, we observed that when either AtAPY1 or AtAPY2 are expressed in plants (Fig. 2.3) or in yeast (Fig. 2.4A) both are processed at their N-termini. The AtAPY1 protein appears to be more effectively processed in both systems. Previously published proteomics data (Dunkley et al, 2004) also indicate that AtAPY1 may be soluble or weakly associated with a membrane. The analysis removed soluble and membrane-associated proteins with Na_2CO_3 and only identified AtAPY2 with other Golgi resident proteins. One explanation to reconcile these conflicts is that the AtAPY1-GFP construct used by Schiller et al. (2012) was driven by the 35S promoter. The cells may be over-loaded with the AtAPY1-GFP and cannot be properly processed. Even if most of the AtAPY1 protein is soluble and could be secreted into the extracellular matrix, given the enzyme activity assay from Schiller et al. (2012), it may not salvage eATP/ADP. These pieces of evidence further indicate the likelihood that the N-termini serve as a signal peptide and are subsequently processed. The most likely scenario is that AtAPY1 and AtAPY2 complex with either glycosyltransferase or the nucleotide sugar transporters and are maintained in the Golgi lumen.

Earlier studies, based on antibody inhibition results, suggested that AtAPY1 and AtAPY2 could be localized on the plasma membrane and regulate the concentration of extracellular nucleotides in cells that release ATP as they expand (e.g. pollen tubes, root hairs, and stomata) (Steinebrunner et al., 2003; Wu et al., 2007 and Clark et al., 2011). Our results do not show any plasma membrane localized AtAPY1 or AtAPY2. A possible explanation for the conflict is that the polyclonal antibodies used in the prior studies, which bind equally well to the nearly identical AtAPY1 and AtAPY2 proteins (Steinebrunner et al., 2000), may not be specific enough to inhibit only the AtAPY1 and AtAPY2 members of the apyrase family in Arabidopsis. There are five more apyrase (AtAPY3 to 7) family members in Arabidopsis, all sharing the five conserved sequences (ACRs) common to all members of the GDA1_CD39 superfamily. Both AtAPY6 and AtAPY7 contain members with two predicted transmembrane domains, a property

characteristic of most cell-surface-localized NTPDases (Zimmermann, 2001; Knowles, 2011). Although the subcellular localization and functions of AtAPY3 to 7 in plants are still unknown, if any are plasma membrane localized ecto-apyrases, their activity could be inhibited by the polyclonal antibodies used in the inhibition studies, and thus cause the increases in the external ATP concentration in the pollen growth medium observed by Wu et al. (2007).

Intracellular nucleoside triphosphate diphosphohydrolases

In *S. cerevisiae*, only two NTPDases (GDA1 and YND1) have been identified within the entire yeast genome (Abeijon et al., 1993; Zong and Guidotti, 1999; Gao et al., 1999). The functions of GDA1 and YND1 are partially redundant. GDA1 has higher GDPase activity compared to UDPase activity, but no activity toward other nucleotides. On the other hand, YND1 has broader substrate specificities, for it can also hydrolyze NTPs. In addition, overexpression of YND1 in $\Delta gda1$ cannot fully recover the glycosylation defect that occurs in invertase or chitinase (Gao et al., 1999). The yeast $\Delta gda1$ and $\Delta ynd1$ mutants also show different drug sensitivities. However, a detailed understanding of how these two enzymes differentially regulate glycan synthesis has not yet been achieved. Here, we show that AtAPY1 and AtAPY2 perform functions similar to GDA1, since expression of AtAPY1 and AtAPY2 in $\Delta gda1$ can rescue the glycosylation defect of CPY1 (Fig. 2.4A). However, in $\Delta ynd1$ cells, no decrease in hygromycin B sensitivity was observed with the expression of either AtAPY1 or AtAPY2. These results again confirm the differential roles of GDA1 and YND1 in yeast (Fig. 2.4A and B).

Furthermore, we confirmed that knocking out both AtAPY1 and AtAPY2 reduces the microsomal UDPase activity significantly when compared with WT. However, only knocking out APY2 can cause drastic reduction of GDPase activity (Fig 2.5). In the R2-4A line of Arabidopsis (Wu et al., 2007), the repression of AtAPY1 expression by RNAi and the APY2 loss-of-function mutation also caused a strong reduction in UDPase/GDPase activity compared to levels in wildtype plants (Fig. 2.5). The C-terminal GFP tagged AtAPY1 and AtAPY2 can restore the UDPase/GDPase activity in the *apy1 apy 2 dKO* lines (Fig 2.5). It is interesting to note that AtAPY1 and AtAPY2 also show

negligible ADPase activity which is similar to their mammalian orthologues NTPDase 5 and 6 (Mulero et al., 1999 ; Hicks-Berger et al., 1999). Neither the *apy1* or *apy2* mutant lines nor their complementation with AtAPY1-GFP or AtAPY2-GFP constructs altered the ADPase activity. These results indicated that neither AtAPY1 nor AtAPY2 function as ADPases.

Transport of Nucleotide sugars

It has been proposed that nucleotide sugars utilized in the Golgi lumen are transported from the cytosol in an antiport manner prior to their utilization as substrates by glycosyltransferases (Reyes and Orellana, 2008). After glycosylation reactions the released NDP is hydrolyzed by the luminal NDPase to NMP, which would then function as an exchange substrate for the import of nucleotide sugars into the Golgi lumen (Liu et al., 2010). In mammalian systems UDP inhibits glycosyltransferase activity while UMP does not (Khatra et al., 1974; Kuhn and White, 1977; Brandan and Fleischer, 1982). However, in yeast $\Delta gda1$ mutants the accumulation of GDP could potentially impair the antiport of GDP-mannose, but concentrations do not appear to be high enough to inhibit mannosyltransferase (Berninsone et al., 1994). In plants, it appears that AtAPY1 and AtAPY2 are functionally redundant since knocking out either gene does not result in a discernible phenotype (Steinebrunner et al., 2003; Wu et al., 2007). Initially, we expected that knocking down *AtAPY1* by RNAi in *apy2* mutants (R2-4A) would inhibit nucleotide sugar transport into Golgi apparatus. However, we observed no significant changes in the uptake of UDP-galactose into Golgi enriched fractions obtained from the R2-4A line when compared to wildtype (Fig. 2.6B). The measurement of the NDPase activity in microsomes from the R2-4A line revealed a residual UDPase activity at nearly 50% of WT (Fig. 2.5). Therefore, it is likely that the decrease in UDPase is not enough to impair the uptake of UDP-galactose measured in vitro. In yeast, absence of both GDA1 and YND1 does not totally abolish nucleotide sugar-dependent protein glycosylation (D'Alessio et al., 2005). In the absence of NMP, transport can still sustain the entrance of nucleotide sugars until inner and outer concentrations reach equilibrium, although the efficiency is only one-third of when preloading occurs with the corresponding NMPs (Puglielli and Hirschberg., 1999; Segawa et al., 2005). Together these data suggest the

nucleotide sugar transport rates into the Golgi may be fairly insensitive to the supply of available NMPs for effective antiport of substrates. Currently, we have only demonstrated this insensitivity for UDP-galactose transport and somewhat surprisingly found that only galactose levels were significantly elevated in cell wall material of all mutants analyzed (*apy1*, *apy2* and R2-4A).

Conclusion

In summary, our data reveal that AtAPY1 and AtAPY2 are Golgi localized nucleotide diphosphatases and also suggest that are likely to have roles in regulating UDP/GDP concentrations in the Golgi lumen.

Materials and Methods

Plant Material and Growth Conditions

Arabidopsis thaliana ecotype Wassiewskija (Ws) plants were used as wildtype in this study. The *Arabidopsis apy1*, *apy2* and apyrase RNAi lines (R2-4A) were developed previously (Steinebrunner et al., 2003; Wu et al., 2007). For matrix polysaccharide composition analysis: seeds were surface sterilized and planted on Murashige and Skoog medium (4.3 g/L Murashige and Skoog salts (MS) (Caisson), 0.5% (w/v) MES, 3% (w/v) Sucrose, and 0.8% or 1.0% (w/v) agar (Sigma), pH 5.7). All plates contained 4 μ M estradiol for RNAi induction in R2-4A plants. Plates were placed upright in a culture chamber and grown at 23 °C under 24-h fluorescent light for 7 days. Whole seedlings were used for the analysis of monosaccharide composition. For microsomes isolation: seeds were cultured in half strength MS (Caisson) with 1 mM MES, 2% sucrose and 4 μ M estradiol (pH 5.9) for 7 days. The cultures were maintained at 24°C under 24 h fluorescent light in an orbital shaker (120 rpm). Whole seedlings were collected for the analysis.

Cloning procedures

Binary vectors containing C-GFP fusion with the *AtAPY1* (At3g04080) or *AtAPY2* (At5g18280) promoter and coding sequences were produced using Gateway technology (Curtis and Grossniklaus, 2003). The *Arabidopsis AtAPY1* genomic region was cloned from –1118 bp upstream of the translation initiation site to the final codon (excluding STOP) by PCR using DNA isolated from *Arabidopsis* (BAC T6K12) with primers ApyraseI-5'pt (5'-TCAAAGGCAAAAGTTGTGGA-3') and ApyraseI-3'4002 (5'-TGGTGAGGATACTGCTTCTAGT-3'). The Apyrase 2 genomic region was cloned using the same approach, from –1008bp upstream of the translation initiation site with primers ApyraseII-5'pt (5'-CAAATTCCTCTCGTCCGGTTGAGTAGTAT-3') and ApyraseII-3'-TGA (5'-CGGTGAGGATACGGCCTCGATGGCGC-3') from *Arabidopsis* (BAC MGR7). All the amplifications were performed using Phusion high-fidelity DNA polymerase (New England Biolabs), and the amplified fragments were

cloned (TA cloning) in the entry vector PC8/GW/TOPO (Invitrogen). The constructs were introduced into pMDC107 vector using the Gateway® LR reaction (Invitrogen).

Generation of the AtAPY1 or AtAPY2 C-terminal tagged GFP plants and co-transformation of organelle specific markers

Since *apy1 apy2 dKO* pollen cannot germinate (Wu et al., 2007), the heterozygous mutant of *APY1apy1 apy2apy2* or *apy1apy1 APY2apy2* was transformed with the vector *pMDC107* containing *PROAPY1:APY1-GFP* or *PROAPY2:APY2-GFP*, respectively, using *Agrobacterium tumefaciens* GV3101 (pMP90) via floral dip (Clough and Bent, 1998). All T1 plants were selected with 25 mg/L hygromycin on germination media. Plants with resistance were selected and transplanted to the soil. T2 seeds from individual T1 lines were screened for complementation. Identification of *apy1apy1 apy2apy2* or *APY1apy1 apy2apy2* containing *PROAPY1:APY1-GFP* and identification of *apy1apy1 apy2apy2* or *apy1apy1 APY2apy2* containing *PROAPY2:APY2-GFP* were first screened with previously described primer sets (Steinebrunner et al., 2003). After the background heterozygosity of *APY1apy1 apy2apy2* was confirmed, the transgenic *APY1-GFP* signal could be distinguished from the native *AtAPY1* by a specific set of primers: Apy1F-1457 (5'-GTTCCCTTTGGAGAATGAGCTCGAG-3') and Apy1R-4011 (5'-CCCAAATTGCCTTCCTCATGGTGAGGATA-3'). The *apy1apy1 APY2apy2-APY2GFP* was screened using the above procedure but using the primer set: Apy2F-3427 (5'-TAAAGCCCCTGCTTCACAAT-3') and Apy2R-4262 (5'-GCTTTGGCAGAAATCAAAGA-3'). The genetic background of T3 was also examined using the primer sets from Steinebrunner et al. (2003). The rescued *apy1 apy2 dKO* mutants lines containing *PROAPY1-APY1:GFP* or *PROAPY2-APY2:GFP* were transformed with mCherry tagged organelle markers for cis-Golgi and plasma membrane (Nelson et al., 2007). The transformants were screened with Basta® (120 mg/L) in the soil. T2 seeds were collected for visualization by confocal microscopy.

Pollen Germination

The pollen germination method followed exactly the protocol published by Johnson- Brousseau and McCormick (2004). The pollen was germinated on the media for 4 to 6 hours before imaging by microscopy.

Microscopy

Images were collected using a Leica SP2 AOBS confocal microscope with excitation 488 nm for GFP and 543 nm for mCherry. Emission was collected at 500 to 534 nm (GFP) and 600 to 679 nm (mCherry). All images were obtained with 10x ocular and 63 × objective magnifications (oil). The pinhole diameter was set at 1 airy unit. Images were processed in Adobe Photoshop (Adobe Systems).

Yeast transformation

The *Saccharomyces cerevisiae* strains BY4741 (WT: *MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*), *Δgda1* (*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *gda1Δ0*) and *Δynd1* (*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *ynd1Δ0*) were obtained from Open Biosystem (Thermo Scientific). The *AtAPY1/AtAPY2* cDNA sequence from the start codon to the terminating codon before the TGA was cloned using the following primers APY1-For (5'-

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGACGGCGAAGCGAGC
GATC-3')/ APY1-Rev (5'-

GGGGACCACTTTGTACAAGAAAGCTGGGTCTGGTGAGGATACTGCTTCTAT-
3') and APY2-For (5'-

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGACGGCGAAGCGTGG
GATTGG-3')/ APY2-Rev (5'-

GGGGACCACTTTGTACAAGAAAGCTGGGTCCGGTGAGGATACGGCCTCGAT-
3'. The PCR products were recombined into pDONR-F1-Zeo (Lalonde et al., 2010)

according to instructions (Invitrogen) and verified by sequencing. The *AtAPY1/AtAPY2* pDONR-F1-Zeo constructs were recombined into a Gateway® modified pDR-Leu vector generated by transferring the pPMA-GW-tADH expression cassette from pDRf1-GW (Loqué et al., 2007) into the YEplac181 shuttle vector (Gietz and Sugino, 1988) for use in complementation analysis. Yeast were transformed with the different plasmids using the

lithium acetate transformation method (Gietz and Woods 2002) and selected on solid medium containing Yeast Nitrogen Base (YNB) without amino acids (Difco 291940; Difco, Detroit, MI) supplemented with 3% glucose and 1X dropout-leucine (CSM-ura; Sunrise Science Products, San Diego, CA) .

Protein Extraction and Immunoblotting

Total protein was isolated from *S. cerevisiae* as described previously (Eudes et al., 2011). The protein was quantified by Bradford assay (Thermo Scientific) (Bradford, 1976). A total of 5 µg soluble protein was mixed with 0.2 M Tris–HCl, pH 6.5, 8% (w/v) SDS, 8% (v/v) β-mercaptoethanol, 40% (v/v) glycerol, and 0.04% (w/v) bromophenol blue and boiled for 5 min. Samples were subjected to 10% SDS-PAGE and blotted onto PVDF membrane. An antibody against Carboxypeptidase Y (Abcam: ab34636) was used at 1:5,000 for assessment of differential glycosylation in *S. cerevisiae*. Heterologously expressed AtAPY1 and AtAPY2 were detected using the Universal antibody (UNI) against the Gateway® attB2 site (Invitrogen), used at 1:20,000 (Eudes et al., 2011), followed by incubation with goat anti-rabbit secondary antibody (1:20,000) (KPL Inc., Gaithersburg, MD) for 1 h. The detection was by chemiluminescence using the KPL Protein Detector LumiGLO Reserve Western Blotting Kit (#54-13-50) (KPL Inc., Gaithersburg, MA).

Arabidopsis total protein was isolated from 0.3 g of 7-day-old seedlings. Tissues were ground in 1 mL extraction buffer: 0.1 M Tris–HCl pH 6.8, 20% glycerol, 5% SDS, 0.01 M MG132, 0.2 M DTT, 2 mM PMSF, and 1X proteinase inhibitors (Roche, complete mini, #11836170001) and boiled for 5 min. A total of 20 ug microsomal protein was mixed with 2X Laemmli Sample Buffer (Biorad #161-0737EDU). All the samples were run on an 10% SDS–PAGE gel and blotted onto PVDF membrane. An antibody against GFP (Abcam: ab290) was used at 1:5,000.

Hygromycin B sensitivity assay

The single colonies of each mutant were isolated and cultured in minimal liquid medium (described in the previous section) overnight. Then the liquid cultures were

serially diluted and spotted on the YPD or the YPD solid medium with 50 µg hygromycin B (Calbiochem).

Microsomal isolation

The plant microsomal isolation procedure was slightly modified from previous methods (Rautengarten et al., 2011). Seven-day-old liquid cultured seedlings were ground in liquid nitrogen and then were re-suspended in extraction buffer (5 mM HEPES, pH 7.1, 400 mM sucrose and 1 mM DTT). To remove plant debris, the lysate was filtered through two layers of Miracloth (Calbiochem) and centrifuged at 3000 g at 4°C. The supernatant was taken and centrifuged at 50,000 g at 4°C for 1 h. The resulting pellet was washed with the extraction buffer and centrifuged at 50,000 g 4°C for another 1 h. The final membrane pellet was re-suspended in 10 mM Tris buffer (pH 7.5) for the NDPase assay.

NDPase assay

A total of 20 µg microsomal protein was incubated in 1 mL reaction buffer (3 mM NDP [Sigma-Aldrich], 3 mM MnSO₄, 30 mM Tris-MES, pH 6.5, and 0.03% (v/v) Triton X) for 1 h at room temperature. The released phosphate was measured using the malachite green phosphate assay kit (ScienCell) with slight modifications which is that 100 µL Malachite green reagent A and B were added to the 50 µL solution. The incubation time was followed as instructed.

Monosaccharide composition of cell walls

Alcohol-insoluble residues were prepared from 7-day-old seedlings according to previous procedures (Smith-Moritz et al., 2012). The samples were analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Dionex) using a CarboPac PA20 column based on previous established protocols (Harholt et al., 2006).

Preparation of Golgi-enriched membrane fractions for transporter assay

Arabidopsis plants grown for 15 days in liquid culture in the presence of 4 μ M estradiol. The plants were then homogenized using a razor blade in 5 mL of 0.5 M sucrose, 0.1 M KH_2PO_4 (pH 6.65), 5 mM MgCl_2 and 1 mM dithiothreitol (freshly prepared). Membranes were separated following the procedure described by Muñoz et al (Muñoz et al., 1996). Briefly, the homogenate was filtered through Miracloth (Calbiochem) and centrifuged at 3,000g for 3 min. The supernatant was then layered on 5 mL of a 1.3 M sucrose cushion and centrifuged at 108,000 g for 90 min. The upper phase was removed without disturbing the interface fraction and sucrose layers of 1.1, 0.7 and 0.25 M were overlaid on the membrane pad. The discontinuous sucrose gradient was then centrifuged at 108,000 g for 90 min. The 0.7/1.1 M interface enriched in Golgi apparatus membranes was collected, diluted and centrifuged separately at 108,000 g for 50 min, the pellet was resuspended in 200 μ L of 0.5 M sucrose, 0.1 M KH_2PO_4 (pH 6.65) and 5 mM MgCl_2 and stored at -80°C until use.

Nucleotide sugar transport assay

Golgi enriched membranes, obtained as described above, were used to perform [^3H]-UDP-galactose uptake assays, based on a filtration method previously described (Liang and Sze 1998). 50 μ g of protein from the Golgi apparatus-enriched membrane fraction were resuspended in a buffer containing 250 mM sucrose, 20mM KCl, 25 mM Tris-HCl (pH 7.0). The reaction was initiated by adding 100 μ L of 100 μ M [^3H]-UDP-galactose (approximately 20 nCi), to reach 1 mL, final volume. Aliquots were taken at different times and filtered through 0.45 μ m cellulose-ester filters (Millipore), previously treated with 250 mM sucrose, 20 mM KCl, 25 mM Tris-HCl (pH 7.0) and 1 mM UDP-galactose. The reaction was stopped by filtering and immediately washed using 5 mL of ice cold 250 mM sucrose, 20 mM KCl, 25 mM Tris-HCl (pH 7.0) and 1 mM UDP-galactose. The filters were air-dried and the remaining radioactivity was measured in a liquid scintillation counter. The uptake of [^3H]-UDP-galactose is reported as cpm of [^3H]-UDP-galactose.

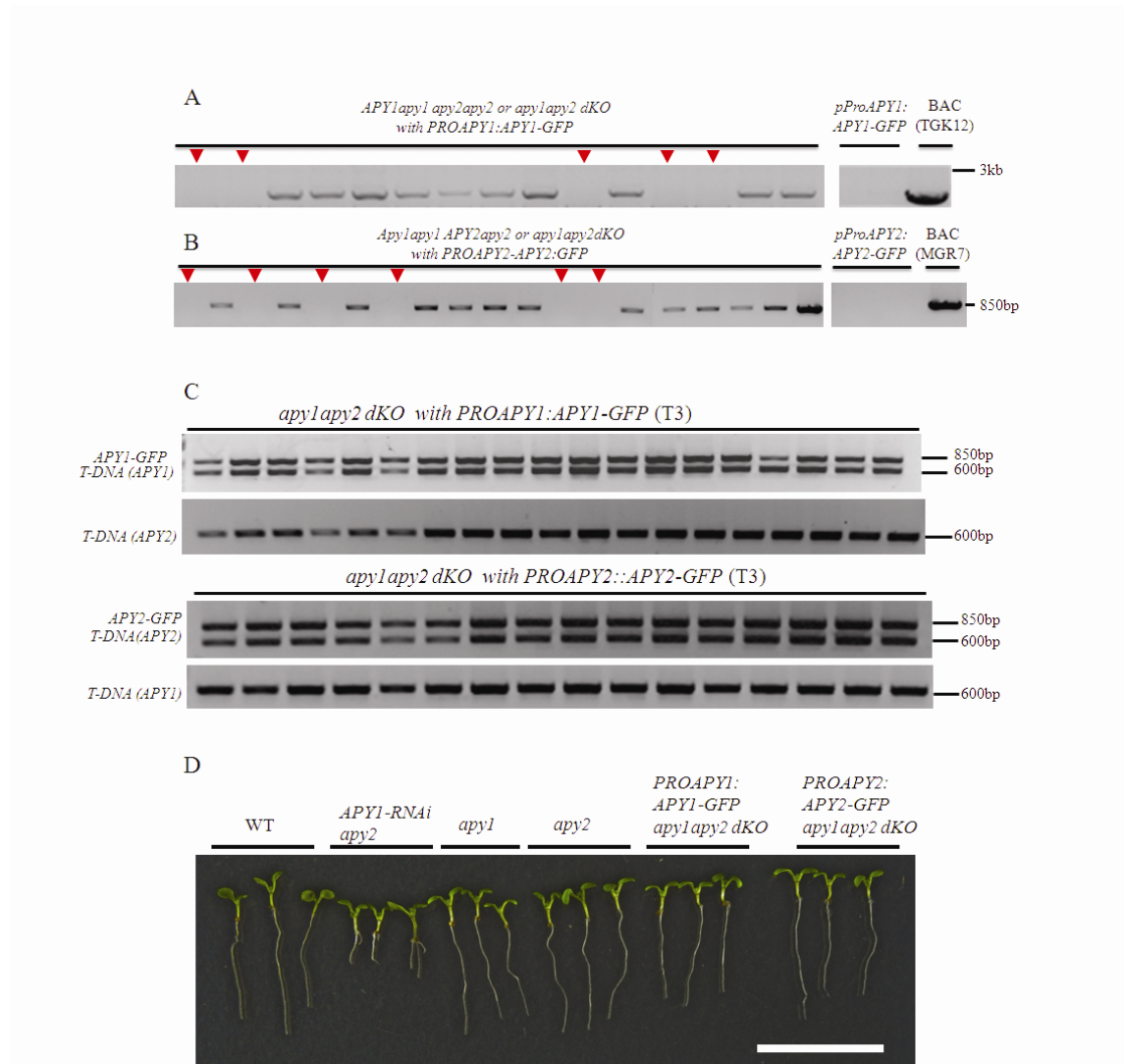


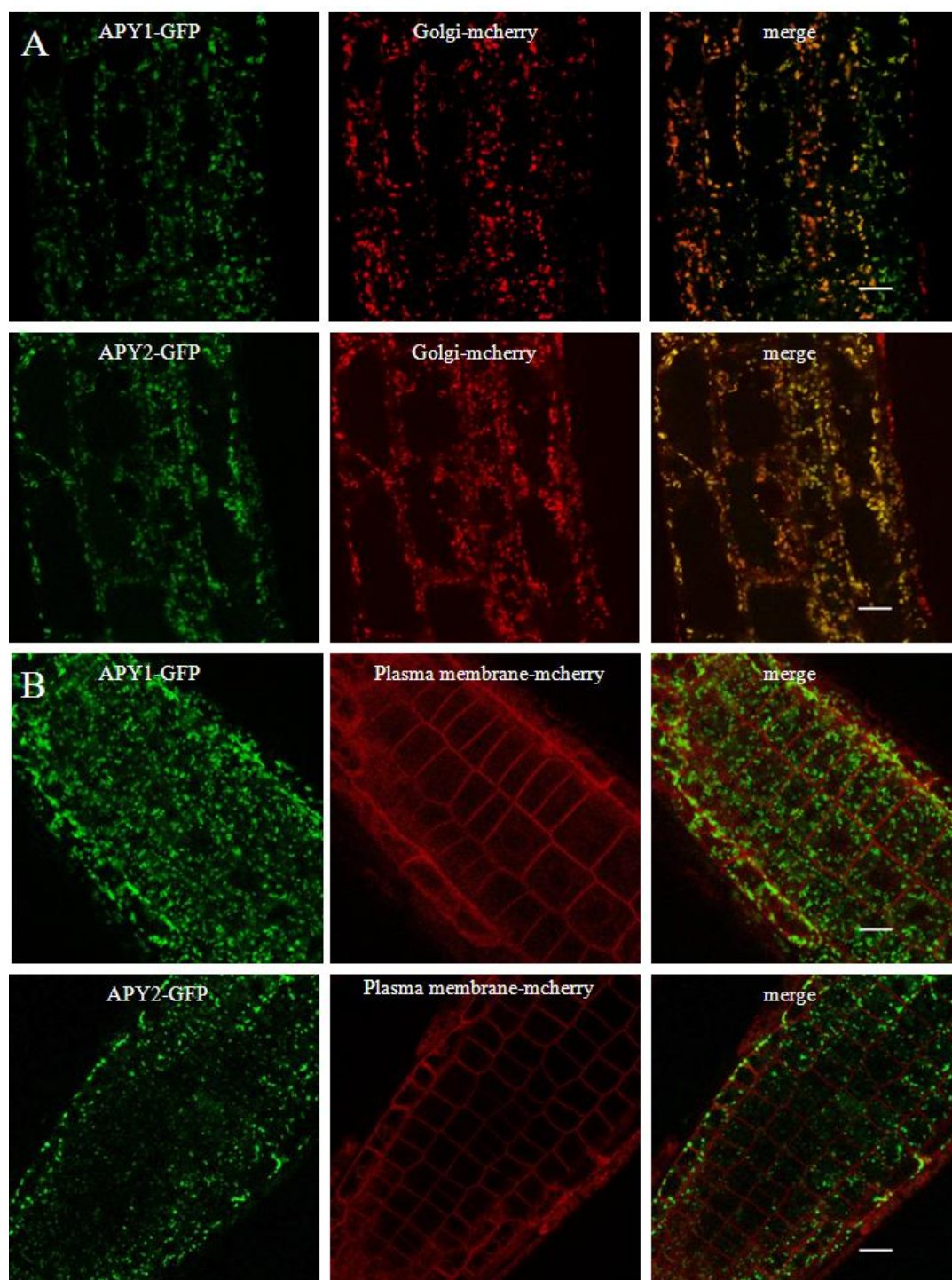
Figure 2.1 Genetic characterization and isolation of *apy1apy2 dKO* plants with either *PROAPY1:APY1-GFP* or *PROAPY2:APY2-GFP* constructs.

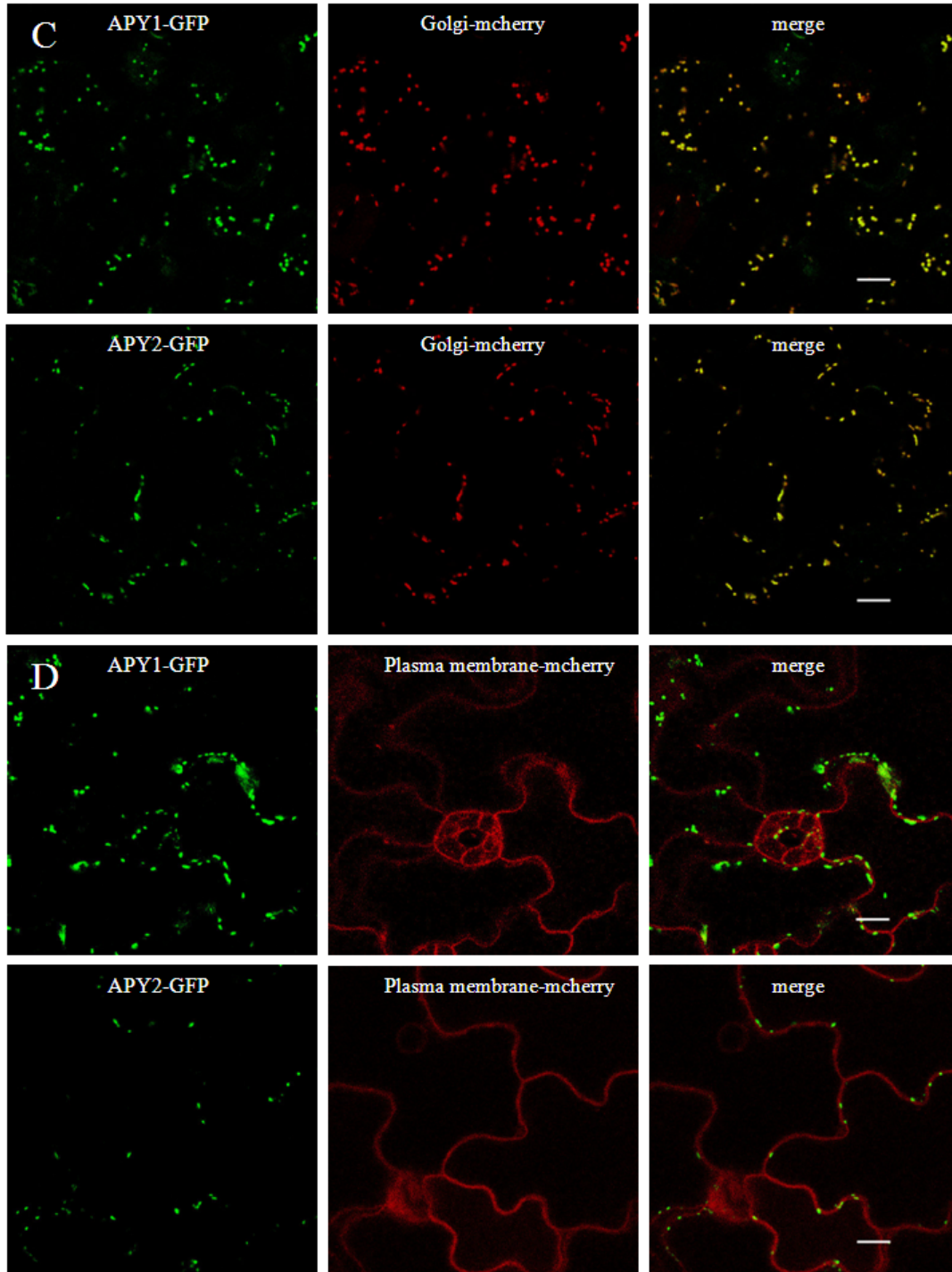
(A) PCR screen of transformed *apy1apy2 dKO* plants (T2) containing the *PROAPY1:APY1-GFP*. The PCR results for *AtAPY1* using primers Apy1F-1457 and Apy1R-4011 from the *PROAPY1:APY1-GFP* transformants indicated that ten lines contained native *AtAPY1* PCR products and five lines did not (▼) resulting in a 2:1 segregation ratio as expected. Lane 1 to 15: genomic DNA isolated from independent *PROAPY1*-*APY1*:GFP lines; lane 16 and 17: vector containing *PROAPY1*-*APY1*:GFP (negative control); lane 18: BAC clone T6K12 containing *AtAPY1* (positive control).

(B) PCR screen of transformed *apy1apy2 dKO* plants (T2) containing the *PROAPY2-APY2:GFP*. The PCR results for *AtAPY2* using primers Apy2F-3427 and Apy2R-4262 from the *PROAPY2-APY2:GFP* transformants indicated that twelve lines contained a native *AtAPY2* PCR products and six lines did not (▼) resulting in a 2:1 segregation ratio as expected. Lane 1 to 19: genomic DNA isolated from independent *PROAPY2:APY2-GFP* lines; lane 20 and 21: vector containing *PROAPY2:APY2-GFP* (negative control); lane 18: BAC clone MGR7 containing *AtAPY2* (positive control).

(C) PCR analysis of genomic DNA isolated from T3 lines indicating that both the T-DNA insertions in *AtAPY1* (amplified using primers ApyF and JL202) and *AtAPY2* (amplified using primers APYR and JL202) were maintained in the T3 populations. The T-DNA insertions did not segregate among individuals which indicates that the T2 are true rescued double mutants containing either the *PROAPY1:APY1-GFP* construct (amplified using primers AraF172 and AraR693) or *PROAPY2:APY2-GFP* construct (amplified using primers APY2I6F and APYR).

(D) 7-day old seedlings of *apy1 apy2 dKO* containing either the *PROAPY1:APY1-GFP* or the *PROAPY2:APY2-GFP* constructs show normal growth and development. The RNAi inducible double knockout line *APY1-RNAi apy2* (R2-4A) highlights the root phenotype when these two genes are perturbed. WT: Wassilewskija; *APY1-RNAi apy2*: R2-4A (Wu et al., 2007); *apy1*: *AtAPY1* single mutants (Steinebrunner et al., 2003); *apy2*: *AtAPY2* single mutants (Steinebrunner et al., 2003).





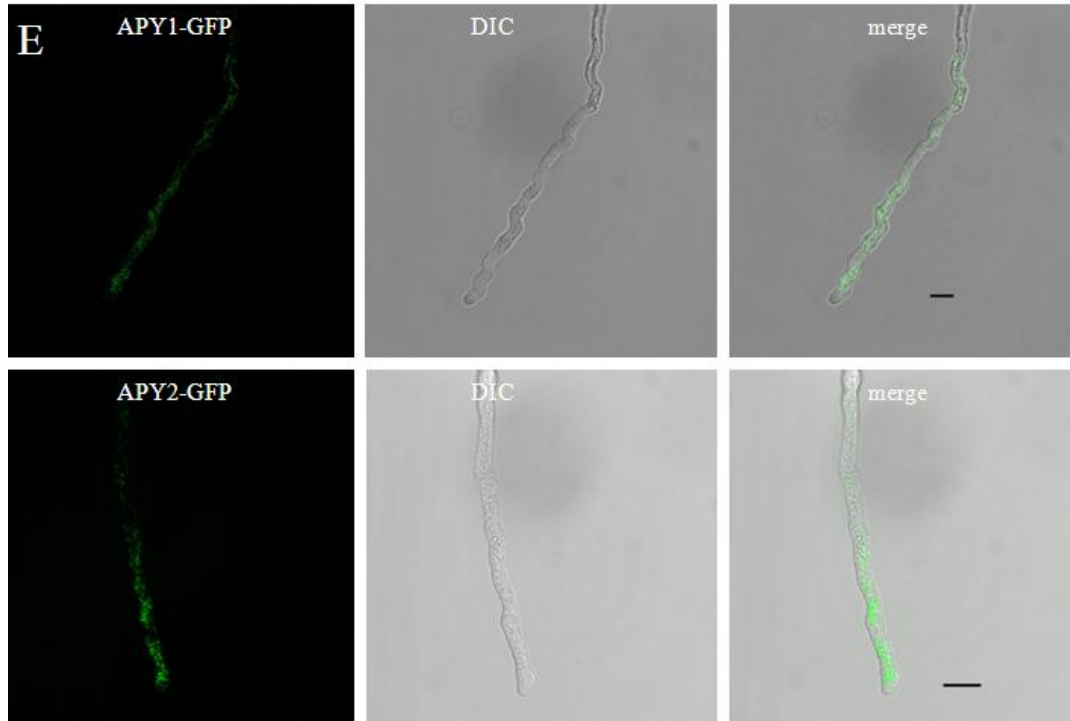


Figure 2.2 Arabidopsis AtAPY1 and AtAPY2 co-localized with the cis-Golgi marker (Man49-mCherry) but not with plasma membrane marker (PIP2A-mCherry) in various tissues. (A) and (B) primary roots of 7-day old seedlings. (C) and (D) cotyledon epidermal cells from 7-day old seedlings. (E) pollen tubes 4 to 6 hours after germination. Bars =10 μ m.

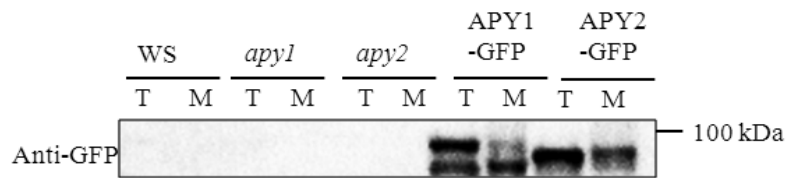


Figure 2.3. Immunoblot analysis of *apy1 apy2 dKO* lines containing AtAPY1-GFP and AtAPY2-GFP constructs.

Total protein (T) and microsomal fractions (M) were isolated from wildtype Arabidopsis (WS), *apy1* and *apy2* mutants and *apy1 apy2 dKO* lines complemented with APY1-GFP and APY2-GFP constructs. Protein lysates (20 µg) were analyzed by immunoblotting using a GFP specific antibody. The entire immunoblot and associated SDS-PAGE gel are outlined in Fig. S3.

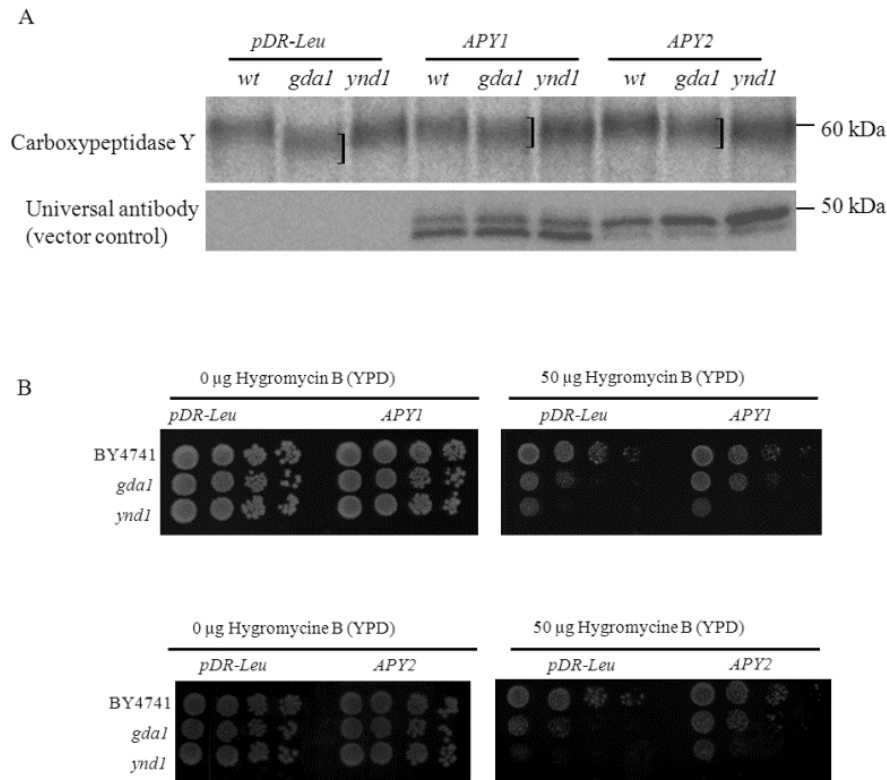


Figure 2.4. Complementation of Golgi-localized yeast apyrases with AtAPY1 and AtAPY2.

(A) The heterologous expression of AtAPY1 and AtAPY2 is capable of rescuing the glycosylation defect in Carboxypeptidase Y demonstrated by a mobility shift in the *Agda1* yeast mutants. Expression of both the AtAPY1 and AtAPY2 could be assessed using the Universal antibody against a Gateway-specific epitope (attB2) at the C-terminal of the construct.

(B) Expression of either AtAPY1 or AtAPY2 in the yeast $\Delta ynd1$ apyrase mutant background. The $\Delta ynd1$ mutant is sensitive to Hygromycin B (50 μ g). Neither Arabidopsis APY constructs could rescue this sensitivity, indicating that they are not functional orthologous of the YND1 type apyrase.

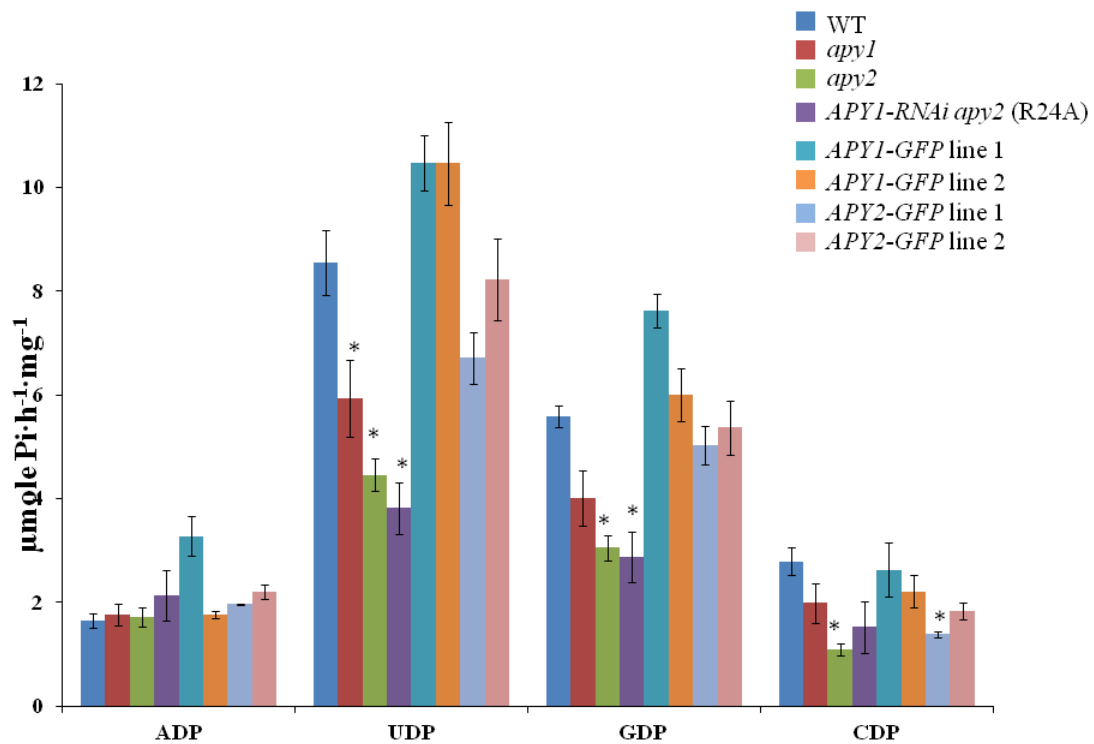


Figure 2.5. Nucleotide diphosphatase activities in the Arabidopsis apyrases mutants. Microsomes were isolated from 7-day old seedlings and NDPase activity ($\mu\text{mole Pi h}^{-1} \text{mg}^{-1}$) measured after incubation for 60 minutes with various NDP substrates ($n = 6$, mean \pm SE). The * indicates significant differences from the WT ($p < 0.05$).

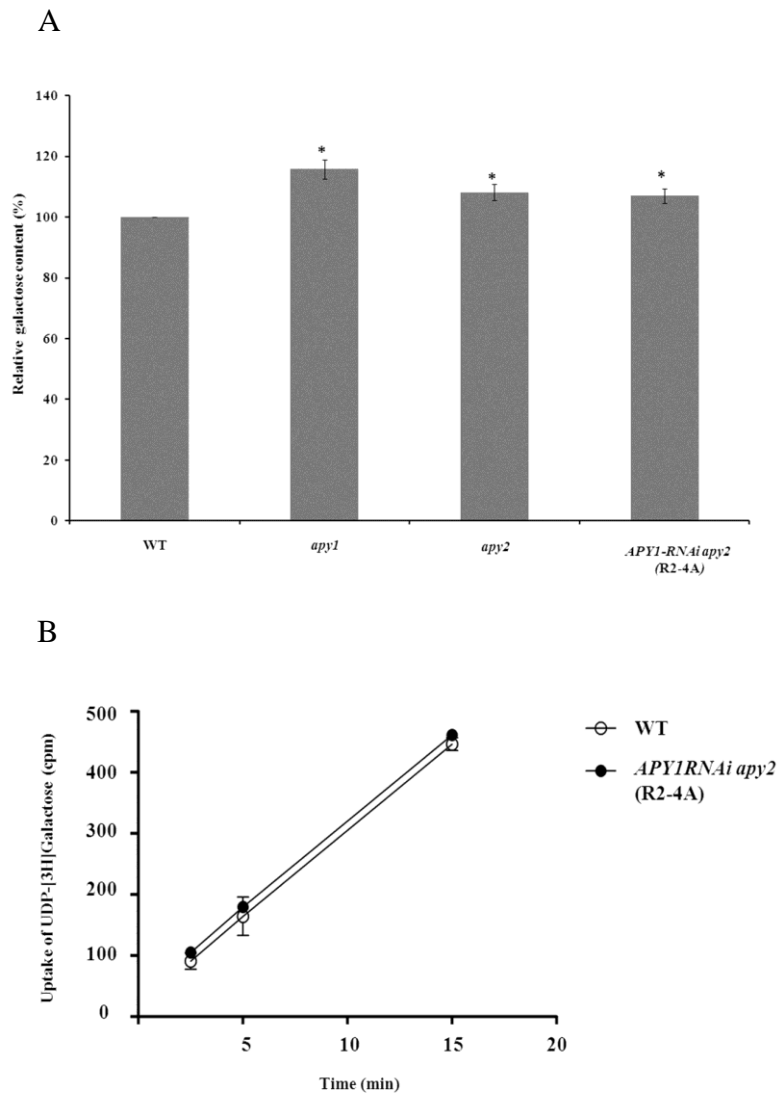


Figure 2.6. The effect of knocking out AtAPY1 and AtAPY2 on monosaccharide composition of cell wall extracts and nucleotide sugar transport.

(A) Cell wall material from 7-day old seedlings was isolated and hydrolyzed with trifluoroacetic acid and analyzed by high-performance anion exchange chromatography (HPAEC-PAD). The galactose content is significantly increased in the cell wall extracts of all Arabidopsis apyrase mutants. Monosaccharide content is expressed as mean mol% from six independent samples ($n = 6$, mean \pm SE). The * indicates significant differences from the WT ($p < 0.05$).

(B) Uptake of UDP-[^3H]-galactose in *APY1-RNAi apy2* (R2-4A) mutant. Golgi enriched fractions were isolated from etiolated cultures of beta-estradiol induced *APY1-RNAi apy2* (R2-4A) line and wild type plants. Uptake assays were performed at different times,

using UDP-[³H] galactose as substrate. Uptake it is reported as counts per minute (cpm). Measurements were done in triplicates and the mean \pm SE is shown.

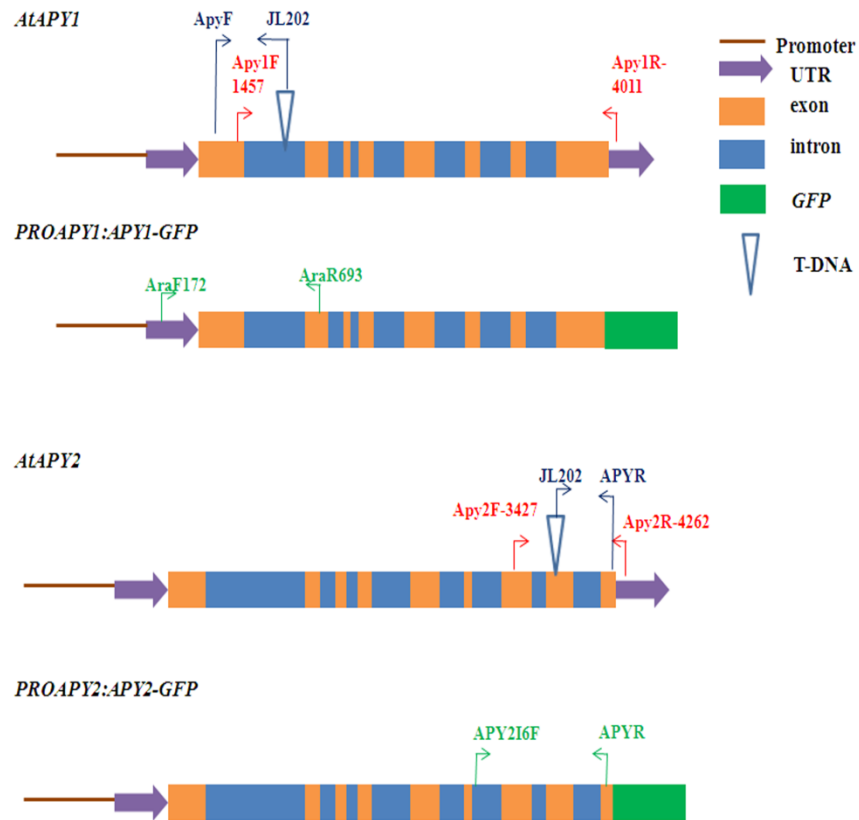


Figure 2.7. Schematic diagrams of *AtAPY1* and *AtAPY2* loci showing T-DNA insertion sites and C-terminal GFP fusion constructs. The location of the primer sets used for screening rescued double mutants is also shown.

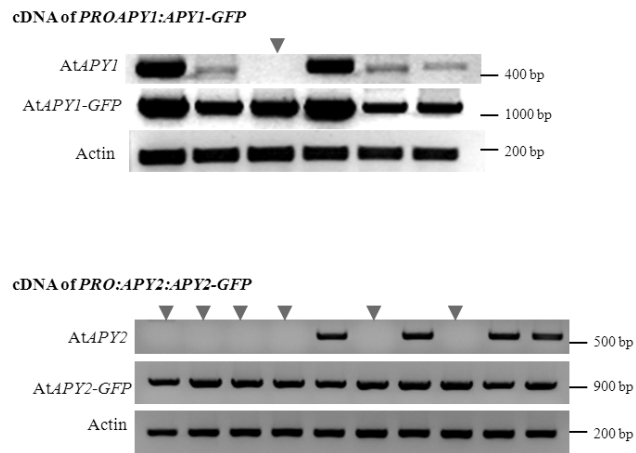
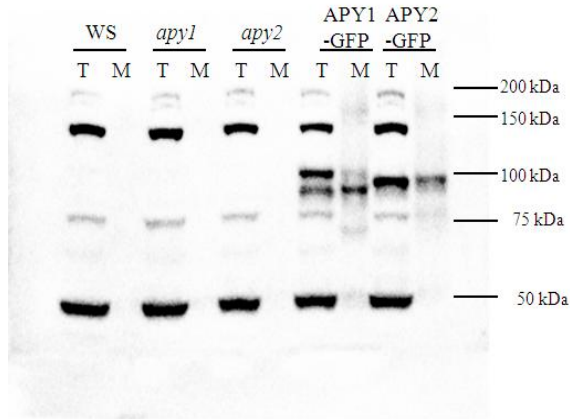


Figure 2.8. RT-PCR of T2 populations to detect native *AtAPY1* or *AtAPY2* transcript. No native *AtAPY1* or *AtAPY2* transcript can be detected in a number of rescued *apy1 apy2 dKO* lines containing *PROAPY1:APY1-GFP* (▼) or *PROAPY2:APY2-GFP* (▼).

A



B

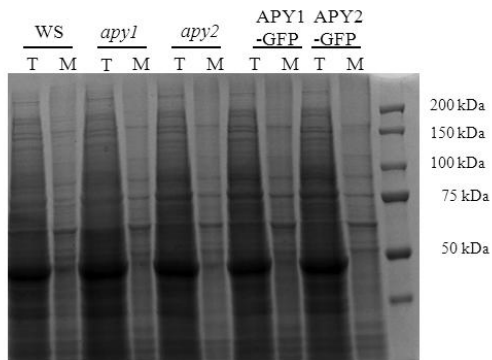


Figure 2.9. Immunoblot and SDS-PAGE of *apy1 apy2* *dKO* lines containing AtAPY1-GFP and AtAPY2-GFP constructs.

(A) The entire immunoblot shown as a cropped image in Fig 2.3. Non-specific bands recognized by the GFP antibody are present in the control samples.

(B) SDS-PAGE demonstrating protein loading.



Figure 2.10. Alignment of primary sequence of AtAPY1 and AtAPY2. The ACRs are shaded in yellow. The CaM-binding domain in APY1 is shaded in pink (Steinbunner et al., 2000). The N terminal transmembrane domain are underlined. The putative cleavage site is indicated with red arrow.

Table 2.1 Monosaccharide composition analysis of wall fractions of apyrase mutants (mol%).

N=6 ; mean±SE

Mol%	Fucose	Rhamnose	Arabinose	Galactose	Glucose	Xylose	GalA	GlcA
WT	1.59 ±0.11	5.91±0.31	11.9±1.14	43.81±2.51	5.44±1.09	9.67±0.52	19.91±0.84	1.72±0.15
<i>apy1</i>	1.81±0.13	5.06±0.39	10.60±1.61	50.76±3.75	4.25±0.41	8.97±0.87	17.09±1.09	1.43±0.08
<i>apy2</i>	1.73±0.14	5.54± 0.36	11.11±1.52	47.71±3.51	3.92±0.23	9.32±0.83	19.03±1.21	1.63±0.086
<i>APY1-RNAi</i> <i>apy2</i> (R2-4A)	1.63±0.12	5.78± 0.28	11.45±1.19	46.7±2.3	4.68±0.13	9.51±0.64	18.7±0.95	1.50±0.05

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Chapter 3. The localization and biochemical characterization of *Arabidopsis thaliana* APY3 through 7

Introduction

NTPDases are enzymes that can hydrolyze both di- and triphosphate nucleotides. They require divalent cations (Mg^{2+} , Ca^{2+}) for activation, and are insensitive to F-type, P-type, and V-type ATPases inhibitors. They are grouped in the GDA1_CD39 superfamily by their five highly similar ACRs (Knowles, 2012). The surface-localized mammalian NTPDases (1, 2, 3 and 8) are known to regulate the availability of extracellular nucleotides such as extracellular ATP (eATP) and ADP (eADP) for binding to and activating the purinoreceptors. In mammalian cells, extracellular ATPs can function as neurotransmitters in the nervous system to depolarize the membrane and send the signals to the neighbor neurons and they can also regulate platelet aggregation in blood vessels (Komoszynski and Wojtczak 1996; Gayle et al., 1999; Buerger et al., 2005). All the surface localized NTPDases carry two transmembrane domains (TMDs) that are important for nucleotide binding and hydrolysis (Grinthal and Guidotti, 2004; 2006 and 2007). In contrast, NTPDases (4, 5, 6, 7 and YND) can also be found intracellularly (Knowles, 2012). The most well characterized intracellular NTPDases are the GDA and YND from *Saccharomyces cerevisiae* (Abeijon et al., 1993). They are responsible for hydrolyzing the NDP from NDP sugars to NMP in the Golgi lumen. The NMPs are then used as an antiport substrate to exchange for more NDP sugars (Abeijon et al., 1993; Berninsone et al., 1994).

In *Arabidopsis*, seven NTPDases have been identified by using the ACRs. APYRASE 1 (AtAPY1: At3g04080) and APYRASE 2 (AtAPY2: At5g18280) are the only two that have been well characterized. These two apyrases both have a hydrophobic segment at the N-terminus. Bacterially-expressed AtAPY1 and AtAPY2 can hydrolyze ATP and ADP but not AMP, while both are insensitive to inhibitors of ATPases (Steinebrunner et al., 2000). Recently Steinebrunner's group showed that plant-expressed APY1-GFP prefers UDP over ATP as its substrate (Schiller et al., 2012). AtAPY1 and AtAPY2 play several physiological roles in pollen development, vegetative growth and stomata opening/closure (Steinebrunner et al., 2003; Wu et al, 2007; Clark et al., 2011). It

has been long argued that AtAPY1 and AtAPY2 may function as cell-surface NTPDases based on antibody data (Wu et al., 2007). However, AtAPY1 and AtAPY2 have been identified in Golgi proteomics databases and shown to function as NDPases (Dunkley et al., 2004; Parsons et al., 2012).

Recently five more apyrases (AtAPY3-7) have been cloned from Arabidopsis. Their biochemical and physiological functions remain elusive. Their initial characterization has been done by Jian Yang (Ph. D. dissertation, University of Texas), who assayed the tissue expression patterns of these apyrases by q-PCR analysis. He found that *AtAPY6* is abundantly expressed in pollen and *AtAPY7* is expressed ubiquitously. Knocking out *AtAPY6* and *AtAPY7* caused late anther dehiscence and low male fertility. The YFP-tagged AtAPY6 showed intracellular punctuate structures. He hypothesized that AtAPY6 and AtAPY7 may also function intracellularly as NDPases and regulate cell wall formation. Unfortunately, knocking out AtAPY 3, 4, 5 simultaneously by RNAi in either wildtype or *apy6* background did not show obvious altered phenotypes (Jian Yang, Ph. D. dissertation, University of Texas, 2011).

In order to learn the subcellular localization and some of the biochemical characteristics of AtAPY3-7, transient expression in onion epidermal cells and heterologous expression in *Saccharomyces cerevisiae* were carried out.

Results

All five of the AtAPY3-7 apyrases contain five typical ACRs. They also share 8 conserved cysteines in their protein sequences that are characteristic of animal intracellular NTPDases (Fig. 3.1). Based on the transmembrane prediction program (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>), the AtAPY3-5 apyrases contain one N-terminal transmembrane domain (TMD), but AtAPY6 and AtAPY7 contain TMDs at the C- and N terminus (Fig. 3.1).

The subcellular localization of AtAPY3 -7

The mammalian NTPDases are associated with the plasma membrane to regulate eATP, or intracellularly with ER or Golgi apparatus to regulate the NDP homeostasis in the lumen. In Chapter 2 and in Schiller et al. (2012) AtAPY1 and AtAPY2 were shown to be Golgi localized. However, the localizations of the other members in Arabidopsis are still unknown. Here we explore their subcellular localization by transiently expressing in onion epidermal cells reporter versions of them that are tagged at their C-terminus with YFP. The AtAPY4-YFP and AtAPY7-YFP apyrases showed intracellular punctate localization patterns that clearly overlapped with the cis-Golgi marker α -mannosidase I (α -ManI). The YFP signal for AtAPY5-YFP partially overlaps with a cis-Golgi marker as well. These data indicate that AtAPY 4, 5, and 7 are probably cis-Golgi resident proteins. However, the intracellular localization patterns for AtAPY3-YFP and AtAPY6-YFP are distinctly different from that of the other YFP-tagged members the family. The fluorescent signal for AtAPY3-YFP displayed a smaller punctate pattern that did not overlap with the cis-Golgi marker. The pattern for AtAPY6-YFP was diffuse and net-like, and it co-localized with the ER marker (Fig. 3.2).

Heterologous expression of APY3-7 in yeast

In order to investigate the biochemical characteristics of AtAPY3-7, Jian Yang attempted the heterologous expression of these five apyrases in *E. coli*, or transiently expressed them in tobacco leaves, but none of these methods yielded successful expression (Ph. D. dissertation, University of Texas, 2011). This result may be attributed to the toxicity of the high expression of these apyrases in bacteria and tobacco leaves.

The bakers' yeast, *Saccharomyces cerevisiae* has two NTPDase orthologues Gda1 and Ynd1 and knockout mutants of these are commercially available. These mutants would be a good system to express the AtAPYs without stressing the organisms. The expressions of APY3-7 in various yeast backgrounds (BY4741, $\Delta gda1$, $\Delta ynd1$) were successfully done with AtAPY 3, 4 and 5. These heterologously-expressed apyrases showed the predicted molecular weight around 50 KD on immunoblots, as detected by their C-terminal attB tags (Fig. 3.3). No expression of AtAPY6 or 7 in yeast could be detected even when more total proteins were loaded or the immunoblot transfers were done with wet transfer apparatus, even though the RT-PCR assays indicated that the transcripts were successfully made in yeast (Fig. 3.4).

AtAPY5 can rescue the Carboxypeptidase Y glycosylation in $\Delta gda1$

Given their subcellular localization, initially I attempted to examine whether all the AtAPY3-7 apyrases can complement the yeast $\Delta gda1$ mutant. To address this point, we expressed each of the five in a wild-type strain of yeast (BY4741) and in $\Delta gda1$ cells. Since no expression of AtAPY6 or AtAPY7 was detected in yeast, this complementation test could not be carried out with these two apyrases. The vacuolar carboxypeptidase Y (CPY) contains four N-linked carbohydrate chains that can be used as an *N*-glycosylation marker in yeast (Stevens et al., 1982). The $\Delta gda1$ mutant is lacking in GDPase activity and impaired in GDP-mannose transport, which results in the CPYs being underglycosylated. In $\Delta gda1$, the underglycosylated CPYs would move faster than the wild-type CPYs in the gel. Based on the amino acid sequences of AtAPY3, 4 and 5, these apyrases show more than 68% similarity to the yeast Gda1 (Fig. 3.1) (Jian Yang, Ph. D. dissertation, 2011). However, their functions associated with regulating the NDP concentrations are quite distinct. Expressing AtAPY5 in $\Delta gda1$ can fully rescue the CPY glycosylation defect, but AtAPY3 cannot. Partial complementation of CPY mobility was observed in $\Delta gda1$ cells expressing AtAPY4.

None of the AtAPY3-5 apyrases can rescue the hygromycin sensitivity of Δynd cells

The other yeast NTPDase, Ynd1, is also intracellularly associated with the Golgi apparatus. Knocking out Ynd1 will both cause cell wall defects in yeast and make the cells more sensitive to hygromycin (Gao et al., 2000). In order to test if any one of the AtAPY3-5 apyrases could have similar functions as Ynd1, $\Delta ynd1$ expressed with AtAPY3, or AtAPY4, or AtAPY5 genes were stressed with 25 μ g/mL hygromycin. None of expressed AtAPY3-5 genes could rescue the hygromycin sensitivity in $\Delta ynd1$, and this suggested the functions of AtAPY3-5 are different from the Ynd1 (Fig. 3.6).

Defect in cell growth due to disruption of GDA and YND can be reverted by APY4 and APY5

A $\Delta ynd1\Delta gda1$ double knockout ($\Delta ynd1\Delta gda1$ dKO) was created by crossing the $\Delta ynd1::URA3$ haploid (XGY4) with the $\Delta gda1::LEU2$ haploid (G2-11). The $\Delta ynd1\Delta gda1$ double knockout cells (KAI1) showed slow growth compared to the single mutant and wild-type strain on a YPAD plate at 30 °C even after 5 days of culture (Gao et al., 1999). However, the lab that created the $\Delta ynd1\Delta gda1$ double knockout lost the original strain. Here, I re-created the $\Delta ynd1\Delta gda1$ dKO by replacing the GDA open reading frame (ORF) with URA ORF by the heterologous exchange in $\Delta ynd1$ single mutant (Baudin et al., 1993 and Wach et al., 1994). The $\Delta ynd1\Delta gda1$ dKO was isolated by using primer sets described in the material and methods. The fact that no GDA ORF sequence but only URA ORF could be detected by PCR indicated that the URA ORF successfully replaced the GDA ORF to disrupt the function of GDA (Fig. 3.7).

The mutant strain I obtained also displayed slow growth as described in the previous publication (Gao et al., 1999) (Fig 8). Heterologously-expressed AtAPY4 and AtAPY5 could rescue the slow growth phenotype in the $\Delta ynd1\Delta gda1$ dKO (Fig. 3.8A and B). Both Golgi-localized NTPDases described in Chapter 2, AtAPY1 and AtAPY2, can also reverse the growth defect of $\Delta ynd1\Delta gda1$ dKO (Fig 8A and 8B). Interestingly, the fact that AtAPY3 cannot complement the $\Delta ynd1\Delta gda1$ dKO may be due to its distinct localization outside the cis-Golgi (Fig 3.2 and Fig. 3.8A and B).

AtAPY 3, 4 and 5 show distinct enzyme activities

The enzyme activities of AtAPY3, 4, and 5 were demonstrated by expressing them in the $\Delta ynd1\Delta gda1$ *dKO* background. AtAPY3 has a strong preference toward NTPs but also with noticeable activities toward ADP and GDP (Fig 3.9). In contrast, AtAPY5 is a stronger NDPase with much less activities to NTPs (Fig 3.9). Neither AtAPY3 nor AtAPY5 can hydrolyze either CMP or GMP (Fig 3.9). No significant NTPase or NDPase activity could be detected in AtAPY4, but this may due to the assay conditions (Fig 3.9).

Discussion

Besides the well characterized AtAPY1 and AtAPY2, there are five more proteins with typical ACRs in Arabidopsis. In this chapter, I report the localization and some of the biochemical characteristics these five *Arabidopsis thaliana* APYRASEs.

AtAPY3-5

AtAPY 3 (AT1g14240), 4 (AT1g14230) and 5 (AT1g14250) are clustered on chromosome 1. These proteins share more than 68% similarity in amino acid sequences. It was hypothesized that these apyrases arose from gene duplication and may have similar functions in cells. Single mutants did not show any drastic phenotypic changes. Simultaneously knocking down AtAPY3, 4, 5 by RNA interference (RNAi) did not show any phenotypes as well (Jian Yang, Ph. D. dissertation, University of Texas, 2011). Based on the heterologous expression of AtAPY3-5 in yeast, AtAPY3 and AtAPY4 were confirmed to have the predicted molecular mass of 53 kDa and 54 kDa, respectively, by western blot analysis in both the total protein and the microsomal fractions (Fig. 3.3 and Fig. 3.8). The predicted molecular mass of AtAPY5 is 53.5 kDa, but the distinct band of the immunoblot analysis is slightly smaller than the predicted (Fig. 3.3 and Fig. 3.8). This difference may due to the mis-annotation of three codons at the exon-intron junctions. It is interesting to note that in the previous chapter and here, I reported that both AtAPY1 and AtAPY2 have one predicted N-terminus TMD that can be processed and may be soluble or weakly associated with a membrane (Fig. 3.8) (Chiu et al., 2012). In contrast, AtAPY3, 4 and 5 have one terminal TMD at their N-terminus that cannot be proteolytically processed, so they would all be predicted to behave as integral membrane proteins (Fig. 3.3 and Fig. 3.8).

In the past knocking out each of the AtAPY3-5 did not result in any drastic phenotypes (Jian Yang, Ph. D. dissertation, University of Texas, 2011). Although the transient localization provides preliminary results about the subcellular locale, these results would be made more definitive by showing that the YFP fusion proteins actually function in the mutants. Since AtAPY3 through 5 are clustered together on chromosome 1, we hypothesized that the appearance of *AtAPY3* through 5 may be due to gene duplication and that these genes may have redundant roles in Arabidopsis. However,

based on their subcellular localizations and complementation assays of their function in yeast, these three APYRASEs are quite distinct from each other.

In yeast, Gda1 and Ynd1 are two known NTPDases that localize at the Golgi apparatus and early Golgi (<http://yeastgfp.yeastgenome.org/>) and that regulate glycan synthesis (Abeijon et al., 1993; Gao et al., 1999). Knocking out Gda1 and Ynd1 would cause defects in glycosylation and result in growth inhibition (Abeijon et al., 1993; Gao et al., 1999). Transiently expressed AtAPY3-YFP showed a smaller punctuate locale than the cis-Golgi markers in the onion-skin cells (Fig. 3.2). It cannot complement either the defect of N-linked glycosylation of CPY in $\Delta gda1$ (Fig. 3.5), or the hygromycin sensitivity of $\Delta ynd1$ (Fig. 3.6). Moreover, the slow growth of the $\Delta gda \Delta ynd$ *dKO* cannot be restored by expressing AtAPY3-YFP (Fig. 3.8). Regarding substrate preferences, generally AtAPY3 hydrolyzes NTPs better than NDPs (Fig. 3.9). The unique localization and enzyme activity of AtAPY3 indicate that AtAPY3 does not have a function similar to GDA and YND.

Among reported intracellular mammalian NTPDases (NTPDase 4, 5, 6 and 7), only NTPDase 7 displays stronger preferences to NTPs (Shi et al., 2001). However, as yet no physiological significance of NTPDase 7 has been reported (Shi et al., 2001; Knowles, 2011). AtAPY4 showed clear residency in the cis-Golgi (Fig. 3.2). However, although it could partially complement the N-linked glycosylation of CPY of $\Delta gda1$ (Fig. 3.5), it could not reverse the hygromycin sensitivity of $\Delta ynd1$ (Fig. 3.6), but it could restore the slow growth of the $\Delta gda1 \Delta ynd1$ *dKO*. The functional complementations presented above are consistent with the hypothesis that AtAPY4 proteins are Golgi resident and have roles in the cis-Golgi. The fact that it only partially rescues the $\Delta gda1$ phenotypes may due to its weak enzyme activities (Fig. 3.9).

The localization of AtAPY5 remains elusive in plants (Fig. 3.2). However, heterologously-expressed AtAPY5 can complement the N-link glycosylation defect of $\Delta gda1$ and also rescue the growth defect of $\Delta gda1 \Delta ynd1$ *dKO* (Fig. 3.6), which may be due to its robust NDPase activities (Fig. 3.9). Compared with AtAPY1 and AtAPY2, which were reported as strong UDPases in plants, AtAPY5 has much broader NDP preferences (Fig. 3.9). Ynd1 has broader spectrum NDP preferences than Gda1, which is a stronger GDPase (Gao et al., 1999). Thus, it seems reasonable to suggest that AtAPY5

may have similar functions as Ynd1. However, based on the hygromycin sensitivity assay, AtAPY5 cannot restore the phenotype (Fig. 3.6).

AtAPY6-7

Transiently expressed AtAPY6-YFP displayed ER localization and AtAPY7-YFP is cis-Golgi associated. We made several attempts to detect AtAPY6 and AtAPY7 expression in yeast, but none of them were successful (Fig. 3.4). According to the transmembrane prediction program (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>), AtAPY6 and AtAPY7 contain TMDs at both their C- and N- termini (Fig. 3.1). Initially, I hypothesized that the two transmembrane domains could have hindered their ability to move from the SDS-PAGE gel to the PVDF membrane in the semi-dry transfer apparatus, which would result in no signal on the western blot. However, switching to the wet transfer apparatus and extending the transfer duration still failed to give the signal. Nonetheless, based on our RT-PCR analysis, the transcripts of AtAPY6 and AtAPY7 are expressed stably in yeast. One possible explanation to reconcile this is that the protein turnover is so fast in yeast that it is difficult to detect the signal.

Materials and Methods

Cloning procedures for heterologous protein expression

The *AtAPY3-7* cDNA sequence from the start codon to the terminating codon before the TGA was cloned using the primers. The PCR products of *AtAPY3-6* were recombined into pDON-Zeo by BP reaction (Invitrogen) and verified by sequencing. The *AtAPY3-6* pDON-Zeo constructs were recombined into a Gateway® modified pDR-Leu vector (Loqué et al 2007) for use in complementation analysis. The *AtAPY7* cDNA was cloned by Jeemeng Lao (JBEI) into pDON-f1-Zeo and was kindly provided by Dr. Heazlewood. The *AtAPY7* pDON-f1-Zeo constructs were recombined into a Gateway® modified pDR-Leu vector by me, using the method described above.

The *AtAPY3-6* pDON-Zeo and *AtAPY7* pDON-f1-Zeo constructs were also recombined into pBullet by LR reaction (Invitrogen). The pBullet is a customized bombardment vector with a Gateway recombination site, C-terminal YFP, and GmMan1::CFP (Nelson et al., 2007).

Primer name	Primers
APY3 CDS F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCatgaca ccggagacggatgctc
APY3 CDS R	GGGGACCACTTTGTACAAGAAAGCTGGGTCAaagccaagata ttttctagat
APY4 CDS F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCatgcaa cggctctaatgctcggtc
APY4 CDS R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTattttgtatttggc tacatta
APY5 CDS F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCatggat gctctcaagggttcaga
APY5 CDS R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCttaagccaatc atttttctag
APY6 CDS F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCatgcg acgatctcatgcccggtc
APY6 CDS R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTctaattcgagtc actatgtaccg
APY7 CDS F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCatggttt ttggtaggatcactga
APY7 CDS R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCattttgagcatgt gtgaatcag

Yeast transformation

The *Saccharomyces cerevisiae* strains BY4741 (WT: *MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*), *Δgda1* (*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *gda1Δ0*) and *Δynd1* (*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *ynd1Δ0*) were obtained from Open Biosystem (Thermo Scientific). Yeast were transformed with the various plasmids using the EZ-YEAST™ transformation kit according to the manufacturer's instructions (MP biomedical) and selected on solid medium containing Yeast Nitrogen Base (YNB) without amino acids (Difco 291940; Difco, Detroit, MI) supplemented with 2% glucose and 1X dropout-leucine (CSM-ura; Sunrise Science Products, San Diego, CA) .

Chromosomal Deletion of the GDA1 Genes

The chromosomal *GDA1* gene was replaced with *URA3* according to the method described previously (Baudin 1993; Wach 1994). The yeast genomic DNA was extracted from *Saccharomyces cerevisiae* wildtype strains BY4741 (WT: *MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*) by YeaStar™ Genomic DNA Kit (Zymoresearch) and used as template. The yeast *GDA1* was cloned by using the primer sets: *pGDA1*(5'-CACCATAAAAAGGGCAAGGACTGC-3') and *tGDA* (5'-ACCTAGCAGTTTATTAAACGACC-3'). The PCR product of full length *GDA* was inserted into the *pENTRY/D-TOPO* (invitrogen) as the templates for creating the knockout cassettes border sequence. Another run of PCR was done by using the primer sets *pGDA1* R (BamHI) (5'-TTCTCGAGAGGATCCCTTAACTG-3') and *tGDA1* R (5'-ATAAGAACATGGACACCGCTTTT-3') to create a BamHI site, which was used to replace *GDA1* ORF with *URA3* ORF. The *URA3* ORF was derived by PCR from *pRS416-GPD* (kindly provided by Dr. Arlen Johnson, University of Texas) and the two cutting sites (BamHI and MscI) were added by using the primer sets: *URA3F* (BamHI CACC) (5'- CACCGGATCCATGTCGAAAGCTACATATAAGGAA-3') and *URA3R* (MscI) (5'- ACAGTTTTGGCCATTAGTTTTGCTGGCCGCATCTTCTC-3'). The *URA3* ORF fragments were digested with BamHI and MscI and ligated into the *pENTRY/D-TOPO-GDA* backbone to create the knockout cassette *pGDA-URA-tGDA*.

To knock out the chromosomal *GDA1* genes, the *Saccharomyces cerevisiae* strains *Δynd1* (*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *ynd1Δ0*) were transformed

with linearized *pGDA1-URA-tGDA1* using Frozen-EZ Yeast Transformation II Kit™ (Zymoresearch). The transformants were selected on solid medium containing Yeast Nitrogen Base (YNB) without amino acids (Difco 291940; Difco, Detroit, MI) supplemented with 2% glucose and 1X dropout-ura (CSM-ura; Sunrise Science Products, San Diego, CA).

Δgda1 Δynd1 dKO screening

Genomic DNA was isolated from the transformants by using YeaStar™ Genomic DNA Kit (Zymoresearch). The internal *GDA* genomic sequence was tested by PCR using the primer sets: left border: *GDA1F* (5'-ATAAAAAGGGCAAGGACTGC-3') and *GDA1R* ORF (5'-CAATGGTATAAGTTTCCTTGG-3'); middle: *GDA1F* ORF (5'-AGAAATTACCGGTTTCGCT-3') and *GDA1R* ORF (5'-CAATGGTATAAGTTTCCTTGG-3') and right border: *GDA3F* ORF (5'-TGGCCCCAAGTTACCTACTG-3') and *GDA1R* (5'-CAAGGGTTATAGAATAGGACA-3'). The inserted *URA* sequence was tested by PCR using the primer sets: *URA3F* ORF (5'-TAAAGGCATTATCCGCCAAG-3') and *URA3R* ORF (5'-AATGCGTCTCCCTTGTCATC-3')

Protein Extraction and Immunoblotting

Total protein was isolated from *S. cerevisiae* as described previously (Eudes et al., 2011). The protein was quantified by Bradford assay (Thermo Scientific) (Bradford, 1976). A total of 5 µg soluble protein was mixed with 0.2 M Tris-HCl, pH 6.5, 8% (w/v) SDS, 8% (v/v) β-mercaptoethanol, 40% (v/v) glycerol, and 0.04% (w/v) bromophenol blue and boiled for 5 min. Samples were subjected to 10 % SDS-PAGE and blotted onto a PVDF membrane. An antibody against Carboxypeptidase Y (CPY) (Abcam: ab34636) was used at 1:5,000 for assessment of differential glycosylation in *S. cerevisiae*. Heterologously expressed AtAPY3-7 were detected using the Universal antibody (UNI) against the Gateway® attB2 site (Invitrogen), used at 1:20,000 (Eudes et al., 2011), followed by incubation with goat anti-rabbit secondary antibody (1:20,000) (KPL Inc., Gaithersburg, MD) for 1 h. The detection was by chemiluminescence using the KPL

Protein Detector LumiGLO Reserve Western Blotting Kit (#54-13-50) (KPL Inc., Gaithersburg, MA).

RNA extraction and RT-PCR

Total RNA was isolated from previously described *S. cerevisiae* strains by using YeaStar™ RNA Kit (Zymo Research). The 500 ng of total RNA was treated with DNase (Invitrogen) and used as template for cDNA synthesis by SuperScript III Reverse Transcriptase (Invitrogen).

Microsomal isolation

The crude yeast membranes were isolated from Δ gdaynd dKO yeast strains carrying plasmids with APYs by disrupting the cells with glass beads in 400 μ L of chilled extraction buffer (20 mM Tris-HCl, 10 mM MgCl₂, 1 mM EDTA, 5% Glycerol, 1 mM DTT, 1 mM PMSF and 1x protease inhibitor cocktail (complete, Roche)). The cells were centrifuged at 5,000g for 10 min at 4 °C and supernatants were collected. The supernatant was then centrifuged at 50,000g for 1 h at 4 °C and the final membrane pellet was re-suspended in 10 mM Tris buffer (pH 7.5) for the NDPase assay.

Measurement of APYRASE activity

A total of 50 μ g microsomal protein was incubated in 1 mL reaction buffer (3 mM NDP/NTP/NMP [Sigma-Aldrich], 3 mM MnSO₄, 30 mM Tris-MES, pH 6.5, and 0.03% (v/v) Triton X) for 1 h at room temperature. The released phosphate was measured using the Malachite green phosphate assay kit (ScienCell) with a slight modification, which is that 100 μ L Malachite green reagent A and B was added to the 50 μ L solution. The incubation time was followed as recommended by the manufacturer.

NTPDase1	-----	
NTPDase2	-----	
NTPDase3	-----	
NTPDase8	-----	
NTPDase4	-----MGRIGISCLFPASWH	15
NTPDase5	-----MATSWGTVFF	10
NTPDase6	-----MKGIRYETSRTSYI	16
NTPDase7	-----MARISFSYLCPASWY	15
APY1	-----MTAKRAIGRHESLADKVH	18
APY2	-----MTAKRGIGRHESLADKIQ	18
APY3	-----MT---	P 3
APY4	-----MQRSNARSRSNINS---	D 15
APY5	-----	
APY6	-----MRRSHARSRVKNSSSSKS	18
APY7	MVFGRITEFTAAASSRLPAGSQSSVPYMPGTGSSPDVGTSVSDSISIGNGGRKNCRLHSAS	60
GDA1	-----MAPIFRNYRFAIGAFV	17
YND1	-----	

NTPDase1	-----MEDTKESNVKTFCSKNILAILGFSSIIAVIALLA	35
NTPDase2	-----MAGKVRSLPPLLLAAAGLAG---LLLLCVP	28
NTPDase3	-----MFTVLTRQPCQAGLKALYRTPTIIALVVLVSVIVLVSITVI	43
NTPDase8	-----MGLSRKEQVFLALLGASGVSGLTALILLLVE	31
NTPDase4	FSISPVGCPRIINTNLQIMVISVLAASVLLYFSVVIIRNKYGRLTRDKKFQRYLARVT	75
NTPDase5	M-----LVVSCVCS-----AVSHRNQQTWFEGIFLSS-	37
NTPDase6	FQQPQHGPWQTRMRKISNHGSLRVAKVAYPLGLCVGVFIYVAYIKWHRATATQAFFSITR	76
NTPDase7	FTVPTV-SPFLR---QRVAFGLGFFISCLLLMLIIDFRHWSASLPRDRQYERYLARVG	70
APY1	R-----HRGLLLVISIPIVLIALVLLMPGTSTSVSVIE-YTMKNH	58
APY2	R-----HRGIILVISVPIVLIGLVLLMPGRSISDSVVEEYSVHNR	59
APY3	ETDALKVQILP-KHQSLP---YTVTKAKSK---SLILLVVSVTITLGLLLYVFNNSNV	55
APY4	MVDPEVQTSPGNHRSSP---STAAKPKSKR-TKSIIIFVIVACVTIALG---LLFIGYSI	68
APY5	-MDALKVQILP-DNQSSPSTHMLTKPKSKKATKSIAMLIVASLAITLG-LLFVFSSNSV	57
APY6	DMDPIKFQIRSGNRAPSSSSTYTLTKPNSKH-AKSNLLLTVGSSISVVLG---VLFLCYSI	74
APY7	LQDFSSYHGFDPEESILPREAISWGQNGSSFSKEKGSVPNGTNPSTRKRLIRAVMIVMCL	120
GDA1	IMLILLIKTSSIGPPSIARTVTPNASIPKTPEDISILPVNDEPGYLQDSKTEQNYPELAD	77
YND1	-----	

		ACR1	CR1		
NTPDase1	GI-----TQNKALPENVKYGIVL	DAGSSHT	SLYIYKWP	AEKENDTGVVHQVEE 83	
NTPDase2	-----TRDVREPPALKYGIVL	DAGSSHT	SMFIYKWP	ADKENDTGIVGQHSS 74	
NTPDase3	QI-----HKQEVLPGLKYGIVL	DAGSSRT	TVVYVQWP	AEKENNTGVVSQTFK 91	
NTPDase8	-----ATSVLLPTDIKFGIVF	DAGSSHT	SLFLYQWL	ANKENTGVVVSQALA 77	
NTPDase4	DI-----EATDTNPNPNVNYGIVV	DCGSSGS	RVFVYCW	PRHNGNPHDLLDIRQM 123	
NTPDase5	-----MCPINVSAS---TLYGIMF	DAGSTGT	RIHVYTF	VQKMPGQ	PILEGEVF 83
NTPDase6	AAPGARWGQQAHSPLGTAADGHEVFYGIMF	DAGSTGT	RVHVVFQ	FTR-PP	RETPTLTHTET 135
NTPDase7	EL-----EATDTEPNLNYGLVV	DCGSSGS	RIFVYFW	PRHNGN	PHDLLDIKQM 118
APY1	-----EGGSNSRGPKNYAVIF	DAGSSGS	RVHVYCF	DNQLDI	VPLENELELF 104
APY2	-----KGGPNRSGPKNYAVIF	DAGSSGS	RVHVYCF	DNQLDI	IPLGNELELF 105
APY3	IS-----SGSLLSRCKRLRYSVLI	DAGSSGT	RVHVFGYW	FESGKPV	DFGKHY 104
APY4	LR-----SGRNR--RVSLHYSVII	DGGSSGT	RVHVFGYR	IESGKPV	DFGGEENY 115
APY5	MF-----SASFL--RRSSLHYSVII	DAGSSGT	RIHVFGYW	FESGKPV	DFGGEHY 105
APY6	LF-----SGGNL--RGSRLYSVVI	DGGSTGT	RIHVFGYR	IESGKPV	FEFRGANY 121
APY7	FLFAFLVYIVS-MYIYTNWSRGASRYVVF	DCGSTGT	RAYVYQAS	INYYKDS	SLPIVMKS 179
GDA1	AVKS-----QTSQTCSEEHKYVIMI	DAGSTGS	RVHIYK	FDVCTSP	PT--LLDEKF 125
YND1	-----MLIENTNDRFGIVI	DAGSSGS	RIHVFK	QDTESL	LHATNQDSQS 44

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CR2

NTPDase1	CRVKG-----P	GISK	FVQKVNEI-GIYLT	EMERAREV	IPRSQH	QETPVY	127		
NTPDase2	QDVPG-----G	GISS	YADNPSGA-SQSLV	GLEQAL	QDVPK	ERHAGTPLY	118		
NTPDase3	CSVKG-----S	GISS	YGNNPDV-PRAFE	EMQKV	KQVPS	HLHGSTPIH	135		
NTPDase8	QQVEG-----P	GISS	YTSNAAQA-GESLQ	GLEEAL	VLIPE	AQHRKTPTF	121		
NTPDase4	RDKNRKPVVM----	KIKP	GIS	EATSPEKV-SDYIS	PLNFA	AHVPRAKHKETPLY	175		
NTPDase5	DSVK-----P	GLSA	FVDQPKQG-AETVQ	GLLEVAKDSIPRSH	WKKT	FPV	126		
NTPDase6	KALK-----P	GLSA	YADDVEKS-AQGI	RELLDVAEKQIP	PFDFW	KATPLV	178		
NTPDase7	RDRNSQPVVK----	KIKP	GIS	AMADTPEHA-SDYLR	PLLSF	AAHV	PVKKHKTPLY	170	
APY1	LQLK-----P	GLSA	YPNDPRQS-ANSLV	TLLDKAEASV	PRELR	PKTPVR	147		
APY2	LQLK-----P	GLSA	YPTDPRQA-ANSLV	SLLDKAEASV	PRELR	PKTHVR	148		
APY3	ANLKL-----T	GLSS	YADNPEGA-SVSV	TKLVEFAKQRI	PKRM	FRRSDIR	149		
APY4	ASLKL-----S	GLSA	YADNPEGV-SES	VTLEVEFAKRV	HKGK	LKKS	DIR	160	
APY5	ASLKL-----S	GLSS	YADNPEGA-SVSV	TKLVEFAKGR	IPK	GKLK	SDIR	150	
APY6	ASLKL-----H	GLSA	FADDPDGA-SVSL	TELEVEFAKGR	VFKGM	WIETEV	166		
APY7	LTEGSRKSRGRAYDRMETEP	GF	DKLVNNTGL-KTAI	KPLIQWAEKQIP	KNAH	RTTSLF	238		
GDA1	DMLEP-----G	LS	FDTSVGA-ANSLD	PLLVAMNYV	PIKARS	CTPVA	168		
YND1	ILQSVPHIHQE-KDWT	FL	KLNPLGLSS	F	EKKPQDAYKSHIK	PLLDFAKNIIP	ESHWS	SCPVF	103

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	ACR2		CR3		ACR3			
NTPDase1	LGATAGMRLRL	MESEELADR	VLD--VVERSLSNY	PFDFQGA	RIIT	GQEEGAYGW	ITINYL	185
NTPDase2	LGATAGMRLRL	NLTNPEASTSVLM--	AVTHLTQY	PFDFRGARILS		GQEEGVFGW	VVTANYL	176
NTPDase3	LGATAGMRLRL	RLQNETAANEVLE--	SIQSYFKSQ	PFDFRGA	QIIS	GQEEGVYGW	ITANYL	193
NTPDase8	LGATAGMRLRL	SRKNSSQARDIFA--	AVTQVLGRS	PVDFWGA	ELLA	GQAE	GAFGWITVNYG	179
NTPDase4	ILCTAGMRIL	PESQQAILEDLL--	TDIPVHF	DFLFSDS	SHAEVIS	GKQEGVYAW	IGIN	233
NTPDase5	LKATAGLRLL	PEHKAKALLFEVK--	EIFRKS	PFLVPKG--SVS	IMDGS	DEGILAW	VTVN	183
NTPDase6	LKATAGLRLL	PGEKAQKLLQKVK--	KVFKAS	PFLVGDD--CVS	IMNGT	DEGVSAW	ITIN	235
NTPDase7	ILCTAGMRLRL	PERKQLAILADLV--	KDLPLEF	DFLFSQS	QAEVIS	GKQEGVYAW	IGIN	228
APY1	VGATAGLRAL	GHQASENIIQAVR--	ELLKGRSRLKTEAN	AVTVLD	GTQEGSYQW	VVTINYL		205
APY2	VGATAGLRAL	GHQASENIIQAVR--	ELLRDRSMLKTEAN	AVTVLD	GTQEGSYQW	VVTINYL		206
APY3	LMATAGMRLLE	VPVQEIQILEVTR--	RVLRSSG-FMFRDE	WANVIS	GSDEGIYSW	ITANYA		206
APY4	LMATAGMRLLE	VPVQEIQILDVTR--	RVLRSSG-FDFRDE	WASVIS	GSDEGVYAW	VVANHA		217
APY5	LMATAGMRLLE	VPVQEIQILDVTR--	RVLRSSG-FKFQDE	WATVIS	GTDEGIYAW	VVANHA		207
APY6	LMATAGMRLLE	VPVQEIKLGVAR--	RVLKSSG-FLFRDE	WASVIS	GSDEGVYAW	VVANFA		223
APY7	VYATAGVRRL	RPADSSWILGNVWS--	ILAKS	PFTCRRE	WVKIIS	GTEEAYFGW	TALNYQ	295
GDA1	VKATAGLRLL	GDAKSSKILSAVRD-HLEKDY	PF	PVVEG	DGV	SMGDEEGVFAW	ITTN	227
YND1	IQATAGMRLRL	PQDIQSSILDGLCQGLKHPAE	FLVEDCSAQIQVID		GETEGLYGW	LGLN	YL	163

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ACR4

NTPDase1	LGKFSQKTR--WFSIVPYETN-----	NQETFGAL	DLGGASTQ	ITFVPQN	QTIESPDN	235	
NTPDase2	LENFIKYG---WVGRWFRP-----	RKGTLGAM	DLGGASTQ	ITFETTS	PAEDRAS	222	
NTPDase3	MGNFLEKN---LWHMWVHPH-----	GVETTGAL	DLGGASTQ	ISFVAGE	KMDLNTSD	241	
NTPDase8	LGTLVKYS---FTGEWQIPP-----	EEMLVGAL	DMGGASTQ	ITFVPGG	PILDKST	226	
NTPDase4	LGRFEHIEDDDEAVVEVNIPGSESSE	AIVRKRTAGILD	DMGGVSTQ	IAYEV	PKTVSFASSQ	293	
NTPDase5	TGQLHGHR-----	QETVGTLD	DLGGASTQ	ITFLPQ	FECTLEQTP	221	
NTPDase6	TGSLKTPG-----	GSSVGMLD	DLGGSTQ	IAFLPR	VEGTLQASP	273	
NTPDase7	LGRFDHEDESDAEATQELAAG-----	RRRTVGIL	DMGGASLQ	IAYE	VPTSTSVLP	280	
APY1	LRTLKGFPY-----	SDTVGVVD	DLGGGSVQ	MAYAI	PEEDAATAPK	243	
APY2	LRNLGKPY-----	SDTVGVVD	DLGGGSVQ	MAYAI	SEEDAASAPK	244	
APY3	LGS LGTDP-----	LETTGIV	ELGGASAQ	VTFVS	SEHVPP----	240	
APY4	LGS LGGEP-----	LKTTGIV	ELGGASAQ	VTFVS	STELVPS----	251	
APY5	LGS LGGDP-----	LKTTGIV	ELGGASAQ	VTFVP	SEHVPP----	241	
APY6	LGS LGGDP-----	LKTTGIV	ELGGASAQ	VTFVS	SEPMP-----	257	
APY7	TSM LGALP-----	KKATFGAL	DLGGSSLQ	VTFENE	ERTHNETN--	333	
GDA1	LGNIGANGP-----	KLPTAAVF	DLGGGSSTQ	IVFE	PTFPINEK	MVD	267
YND1	YGHFN	DYNP-----	EVSDHFTFGFM	DMGGASTQ	IAFAPHDS	GGEIARHR	206

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CR4

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NTPDase1 -----ALQFRLYGKDYNVYTHSFLCYGKDDQALWQKLA-----KDIQV 272
NTPDase2 -----EVQLHLYGQHYRVYTHSFLCYGKDDQVLQRLLA-----SALQT 259
NTPDase3 -----IMQVSLYGYVYTLYTHSFQCYGKNEAEKKFLA-----MLLQN 278
NTPDase8 -----QADFRLYGSDYSVYTHSYLCFGKDDQMLSRLLV-----GLVQS 263
NTPDase4 QEEVAKNLLAEFNLGCDVHQTEHVYRVYVATFLGFGGNAARQRYED-----RIFAN 344
NTPDase5 RG-----YLTSEFMFNSTYKLYTHSYLGFGLKAARLATLG-----ALETEG 262
NTPDase6 PG-----YLTALRMFNRTYKLYSYSLGLGIMSARLAILG-----GVEGQP 314
NTPDase7 QEEAAKILLAEFNLGCDVHQTEHVYRVYVTTFLGFGGNFARQRYED-----LVLNE 331
APY1 PVE-----GEDSYVREMYLKGRKYFLYVHSYLHYGLLAARAEILK-----VSEDSN 289
APY2 PLE-----GEDSYVREMYLKGRKYFLYVHSYLHYGLLAARAEILK-----VSEDSE 290
APY3 -----EYSRTIAYGNISYTIYSHSFLDYGKDAALKKLL----EKL--QNS-AN 281
APY4 -----EFSRTLAYGNVSYNLYSHSFLDFGQDAAQEKLS----ESL--YNSAAN 293
APY5 -----EFSRTISYGNVSYTIYSHSFLDFGQDAAEDKLL----ESL--QNSVAA 283
APY6 -----EFSRTISYGNVSYNLYSHSFLHFGQNAAHDKLWGSLLSRD--HNSAVE 303
APY7 -----LNLRIKSVNHHLSAYSLAGYGLNDAFDRSVVHLLKKLPNVKNKSDL 378
GDA1 G-----EHKFDLKFGDENYTLKQFSLHLYGLKEGRNVNS-----VLVEN 307
YND1 DDIAT-----IFLRVNGDLQKWDVFVSTWLGFGANQARRRYLA-----QLINT 250
. : : *

NTPDase1 ASNE-ILRDPFHPGYKKVVNVSDLYKTPCT-KRFEMT--LPFQQFEIQGIGNYQQCHQS 328
NTPDase2 HG-----FHPGWPRGFSTQVLLGDVYQSPCT-MAQRPQNFNSSARVSLSGSSDPHLQRDL 313
NTPDase3 SPTKNHLTNPCYPRDYSISFTMGHVFDSLCT-VDQRPESYNPNVDVITFEGTGDPSLCKEK 337
NTPDase8 RPAA-LLRHPYLSGYQTTLALGPLYESPCV-HATPP--LSLPQNLTVEGTGNPGAVSA 319
NTPDase4 TIQK-----NRLLGKQTGLTPDMPYLDPLPLDIKDEIQNGQTIYLRGTGDFDLQRET 398
NTPDase5 T-DGHTFRSALLPRWLEAEWIFGG-----VKYQYGGN-----QEGEVGFEPYAE 306
NTPDase6 AKDGKELVSPILSPSFKGEWEHAE-----VTYRVSGQ-----KAAASLHELCAAR 359
NTPDase7 TLNK-----NRLLGKQTGLSPDNPFLLDPLPVGLTDVVERNSQVLHVRGRGDWVSGAM 385
APY1 N-----PCIATGYAGTYKYGG-----KAFKAAAS-----PSGASLDECRRVA 326
APY2 N-----PCIVAGYDGMKYKYGG-----KEFKAPAS-----QSGASLDECRRIT 327
APY3 STVDGVVEDPCTPKGYIIDTNSKN-----YSSGFLADESKLKGSLL--QAAGNFSKCRSAT 334
APY4 STGEGIVDPDCIPKGYIILETNLQK-----DLPGLADKKGKFTATL--QAAGNFSECRSAA 346
APY5 STGDGIVEDPCTPKGYIIDTHSQK-----DSSGFLSEESKFKASLQVQAAGDFTKCRSAT 338
APY6 PTREKIFTDCAPKGYNLDANTQK-----HLSGLLAEESRLSDSF--QAGGNYSCCRSAA 356
APY7 IEGKLEMKHPLNSGYNGQYICSQ-----CASSVQGGKKKSGSVSIKLVGAPNWGECSAL 433
GDA1 ALKDGKILKGDNTKTHQLSSPLPPKVNATNEKVTLESKETYTIDFIGPDEPSGAQCRFL 367
YND1 LPEN-----TNDYENDDFSTRNLNDFMPRGSSDTDFEFKDTIFHIAGSGNYEQCTKS 302

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NTPDase1 ILELFNTSYCP-YSQAFNGIFLPLQG-----DFGAFAFYFVMK-FLNLTSEK--- 376
 NTPDase2 VSGLFSFSSCP-FSRCSFNGVFPVPVAG-----NFVAFSAFFYTVD-FLRTSMGLP-- 362
 NTPDase3 VASIFDFKACHDQETCSFDGVYQPKIKG-----PFVAFAGFYITAS-ALNLSGSFS-- 387
 NTPDase8 IRELFNFSSCQQEDAFDGVYQPPLRG-----QFYAFSNFYITFH-FLNLTSTRQP-- 369
 NTPDase4 IQPFMNKTNET---QTSNLGVYQPIHFQNS---EFYGFSEFYICTEDVLRMGGDYN-- 449
 NTPDase5 VLRVVRGKLHQPEEVQRG-----SFYAFSYYDRAVDTDMIDYEK-GG 348
 NTPDase6 VSEVLQNRVHRTTEEVKHV-----DFYAFSYYDLAAGVGLIDAEK-GG 401
 NTPDase7 LSPLLARSNTS---QASLNGIYQSPIDFNNS---EFYGFSEFFYCTEDVLRIGGRYH-- 436
 APY1 INALKVNSLCTHMKCTFGGVWNGGGGGGQK---KMFVASFFFDRAAEAGFVDPNPVA 382
 APY2 INALKVNDTLC THMKCTFGGVWNGGRGGGQK---NMFVASFFFDRAAEAGFVDPKQVPA 383
 APY3 FALLKEGKENCLYEHCSIGSTFTPDLOG-----SFLATASFYITAKFFELEEKGW--- 384
 APY4 FAMLQEEKGKCTYKRCSIGSIFTPNLQG-----SFLATENFFHTSKFFGLGEKEW--- 396
 APY5 LAMLQEGKENCA YKCSIGSTFTPNIQG-----SFLATENFFHTSKFFGLGEKEW--- 388
 APY6 LTILQDGNKCSYQHC SIGSTFTPKLRG-----RFLATENFFYTSKFFGLGEKAW--- 406
 APY7 AKNAVNSSEWSNAKHGVDCDLQPCALPDGYPRPHGQFYAVSGFFVYRFFNLSAEAS--- 490
 GDA1 TDEILNKDAQCSPPCSFNGVHQPSLVRTFKESN-DIYIFSYFYDRTRPLGMPLSFTLNE 426
 YND1 IYPLLLKNMPCDDEPCLFNGVHAPRIDFAN--D-KFIGTSEYWYTANDVFKLGGEYN-- 356

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NTPDase1 -VSQEKVTEMMKFC AQPWEEIKTSYAGV-----KEKYLSEYCFSGTYILSLLQGYHF 429
 NTPDase2 VATLQQLEAAAVNVQNTWAQIQARVPG-----QRARLADY CAGAMFVQQLSRGYGF 415
 NTPDase3 LDTFN---SSTWNFC SQNWSQLPLLPKF-----DEVYARSYCF SANYIYHLFVNGYKF 438
 NTPDase8 LSTVN---ATIWEFC QRPWKLVEASYPG-----QDRWLRDY CASGLYIILTLHEGYGF 419
 NTPDase4 AAKFT---KAAKDYCATKWSILRERFDRGLYASHADLHRLKYQCFKSAWMFEVFRHGFSGF 506
 NTPDase5 ILKVEDFERKAREVCNLENFTSG-----SPFLCMDLSYITALLKDGFGF 393
 NTPDase6 SLVVGDFEIAAKYV CRTLETQPQS-----SPFSCMDLTYVSLLLQF-FGF 445
 NTPDase7 GPTFA---KAAQDYC GMAWSVLTQRFKNGLFSSHADHRLKYQCFKSAWMYQVLHEGFHF 493
 APY1 EVRPLDFEKAANKACNMRMEEGKSKFPRV-----EEDNLPYLCLDLVYQYTLVDGFGF 436
 APY2 TVRPMDFEKAANKACSMKLEEGKSTFPLV-----EENLPLYLCLDLVYQYTLVDGFGF 437
 APY3 ---LSELIPAGKRYCGEEWSKLILEYPT-----DEEYLRGYCF SAAYTISMLHDSLGI 435
 APY4 ---LSEMILAGKRCFGEWSKLKVYPTF-----KDENLLRYCF SsayiISMLHDSLGV 447
 APY5 ---LSEMILAGKRCFGEWSKLKEKYPT-----KDKYLHRYCF SsayiISMLHDSLGV 439
 APY6 ---LSNMISAGERFCGEDWSKLVRKDPST-----HEEDLLRYCF SsayiIVSLHDTLGI 457
 APY7 ---LDDVLEKGRFC DKAWQVARTSVSPQ-----PFIEQYCF RapyiVSLRGLYI 539
 GDA1 LNDLARIYCKGEETWNSVFSGIAGSLDELE-----SDSHFCLDL SFQVSLHTGYDI 478
 YND1 ---FDKFSKSLREFCNSNWTQILANSDKGVYN-SIPENFLKDA CFKGNWVLNILEHEGFD 412

* : :: .

ACR5

NTPDase1 TADSWEHIHFIGKIQ-----GSDAGWTLGYMLNLTN----- 460
 NTPDase2 DERAFGGVIFQKKA-----DTAVGWALGYMLNLTN----- 446
 NTPDase3 TEETWPQIHFEKEVG-----NSSIAWSLGYMLSLTN----- 469
 NTPDase8 SEETWPSLEFRKQAG-----GVDIGWTLGYMLNLTG----- 450
 NTPDase4 PVN-YKSLKTALQVY-----DKEVQWTLGAILYRTR----- 536
 NTPDase5 ADSTVLQLTKKVNN-----IETGWALGATFHLLQ----- 422
 NTPDase6 PRSKVLKLRKIDN-----VETSWALGAIFHYID----- 474
 NTPDase7 PYD-YPNLRTAQLVY-----DREVQWTLGAILYKTR----- 523
 APY1 KPSQTITLVKKVKYGDY-----AVEAAWPLGSAIEAVS----- 469
 APY2 EPSQTITLVKKVKYGDQ-----AVEAAWPLGSAIEAVS----- 470
 APY3 ALD-DESITYASKAGEK-----HIPLDWALGAFILDVV----- 467
 APY4 ALD-DERIKYASKAGEE-----DIPLDWALGAFILNTA----- 479
 APY5 ALD-DERIKYASKAGE-----NIPLDWALGAFILNTD----- 471
 APY6 PLD-DERIGYANQAG-----DIPLDWALGAFIQQTA----- 487
 APY7 TDKQIIIGSGSI TWTLGVALLESGKALSSTLGLKSYETLSMKIN-----PIAL 587
 GDA1 PLQRELRTGKKIANK-----EIGWCLGASLPLLK----- 507
 YND1 PRIDVDAENVNDRPLFQSVKEVEERELSWTLGRILLYASGSILAGNDDFMVGIAPSERRT 472

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NTPDase1	-----MIPAEQP-LSTPLSHS-----TYVFLMVLFSLVLFVTAIIGLLIFHKPSYFWK	507
NTPDase2	-----LIPADPPGLRKGTDFS-----SWVLLLLFASALLAALVLLLRQVHSAKLPST	494
NTPDase3	-----QIPAESPLIRLPIEPP-----VFVGTLAFFTAALLCLAFLAYLCSATRKRKH	517
NTPDase8	-----MIPADAPAQWRAESYG-----VWVAKVVMVLALVAVVGAALVQLFWLQD---	495
NTPDase4	-----FLPLRDIQQEAFRASHTHWGVSFVYNHYLFSGCFLVVLLAILLYLLRLRRIHR	590
NTPDase5	-----SLGISH-----	428
NTPDase6	-----SLNRQKSPAS-----	484
NTPDase7	-----FLPLRDLRQEGVRQAHGWSFRLSFVYNHYLFFACILVVLLAIFLYLLRLRRIHH	577
APY1	-----SP-----	471
APY2	-----SP-----	472
APY3	-----TPNSDYNGKS-----RKYLGF-----	483
APY4	-----TATFDYSGKS-----RKILDLSNVAKYKI-----	503
APY5	-----TPTSDYNGKS-----RKMIGFK-----	488
APY6	-----TETSQHAASGNLHWFHALFSNHPKTLHYLIGIPILMTVLVYLVTWKRPQLKTI	541
APY7	ISILILSLLLLCALSRVSNCLPRFFRKSYLPLFRHNSTASSVLNIPSPFRFQRWSPMS	647
GDA1	-----ADNWKCKIQSA-----	518
YND1	KLTGKKFIPGKLLESDQLRKQSSSLSNKGFLMWFAIICCFYLIFFHRSHIIRRRFSGLYN	532
NTPDase1	DMV-----	510
NTPDase2	I-----	495
NTPDase3	SEHAFDHAVDSD-----	529
NTPDase8	-----	
NTPDase4	RTPRSSS-AAALWMEEGLPAQNAPGTL-----	616
NTPDase5	-----	
NTPDase6	-----	
NTPDase7	RQTRASAPLDLLWLEEVVPMMGVQVGP-----	604
APY1	-----	
APY2	-----	
APY3	-----	
APY4	-----	
APY5	-----	
APY6	YDLEKGRYIVTRIR-----	555
APY7	TGVKTPLSPTVRGSPRRPFSFGSSIQLMESSLYSSSSCMHSCSSDSLGIQYDSTGSEFW	707
GDA1	-----	
YND1	ITKDFKTGIRRLKFLRRSDPFSRLEEGELGTDVDGFKDVYRMKSSSMFDLGKSSATMQR	592
NTPDase1	-----	
NTPDase2	-----	
NTPDase3	-----	
NTPDase8	-----	
NTPDase4	-----	
NTPDase5	-----	
NTPDase6	-----	
NTPDase7	-----	
APY1	-----	
APY2	-----	
APY3	-----	
APY4	-----	
APY5	-----	
APY6	-----	
APY7	SSPRRSQMRLQSRRSQSRELDSSSLADSHMLKM-----	740
GDA1	-----	
YND1	EHEPQRTASQSANLAPSNI LRPAFSMADFSKFKDSRLYD	630

Figure 3.1. Alignment of primary sequence of human NTPDase 1-8 and Yeast GDA1 and YND1 with AtAPY3-7. Sequence alignment was done by using ClustalW (<http://www.ebi.ac.uk/clustalw/>). The ACRs are shaded in yellow. The conserved regions (CRs) are shaded in different color to show similarities. The conserved cysteine residues are shaded in red.

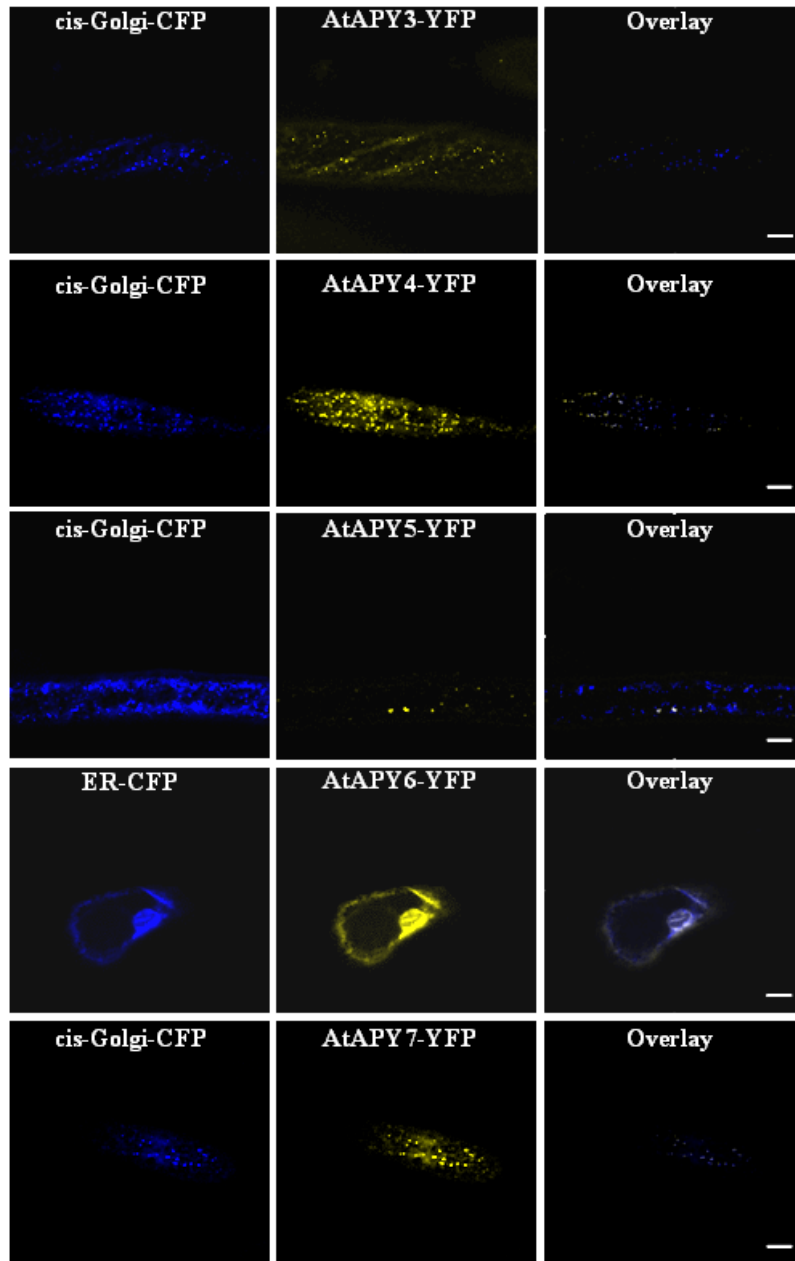


Figure 3.2. Subcellular localization of AtAPY3-7 by their C-terminal YFP tag in onion epidermal cells. Onion epidermal cells were biolistically bombarded with vectors that carried organelle-specific markers fused with cyan fluorescence protein (CFP) and AtAPYs fused with yellow fluorescence protein (YFP). α -mannosidase was used as the cis-Golgi marker. YFP fused with C-terminal HDEL was used as the ER marker. Scale bar = 20 μ M.

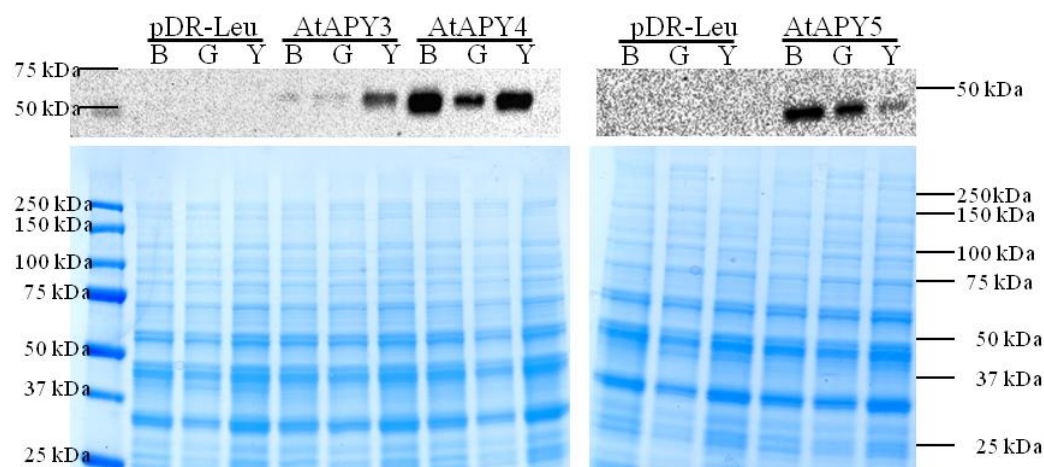


Figure 3.3. The heterologous expression of AtAPY3-5 proteins in various yeast backgrounds. B: BY4741 (wildtype); G: $\Delta gda1$; Y: $\Delta ynd1$. Yeast cells hosting pDR-Leu empty vectors were used as control. Upper panel: Expression of APYs could be assessed by immunoblot analysis using the Universal antibody against a Gateway-specific epitope (attB2) at the C-terminal of the construct. Lower panel: Coomassie blue staining to display equal loading.

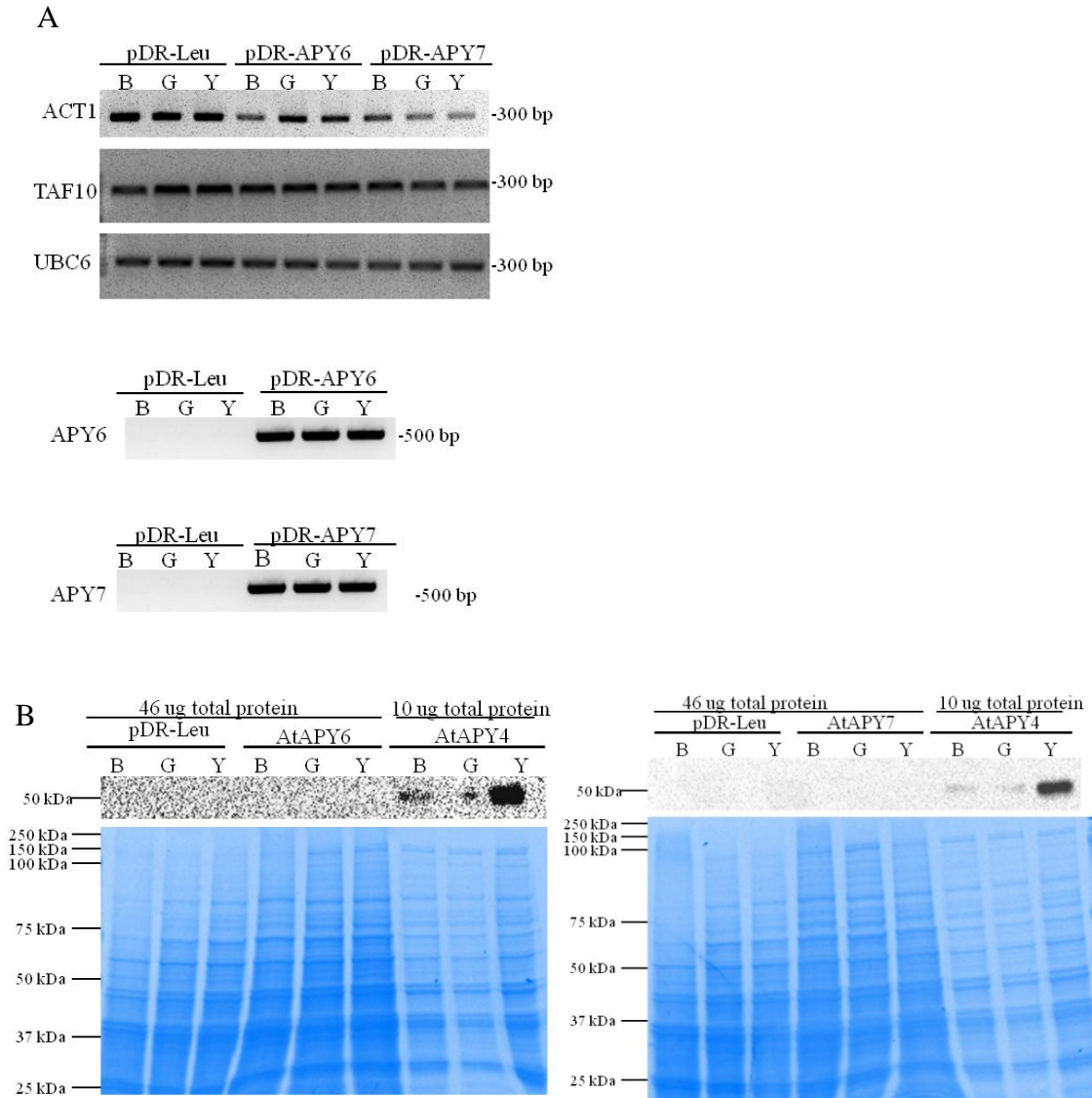


Figure 3.4. The heterologous expression of *AtAPY6* and 7 in various yeast backgrounds.

(A) An RT-PCR analysis of *AtAPY6* and *AtAPY7* transcript abundance in B, BY4741; G, $\Delta gda1$ and Y, $\Delta ynd1$. Upper panel: The house keeping genes *ACT1*, *TAF10* and *UBC6* served as references for the RT-PCR. Lower panel: The transcript abundance of *AtAPY6* and *AtAPY7* as expressed in various cells that were transformed with expression vectors.

(B) The expression of *AtAPY6* and *AtAPY7* as detected by immunoblot in B, BY4741 (wildtype); G, $\Delta gda1$, and Y, $\Delta ynd1$. Yeast cells hosting pDR-Leu empty vectors were

used as control. Upper panel: Expression of APYs was assessed using the Universal antibody against a Gateway-specific epitope (attB2) at the C-terminus of the construct. To detect AtAPY6 and AtAPY7 proteins more total protein of crude yeast extract was loaded to test the possibility of low protein expression of these two apyrases. The APY4 expressed in various backgrounds was loaded as a positive immunoblot control. Lower panel: Coomassie blue staining to display equal loading.

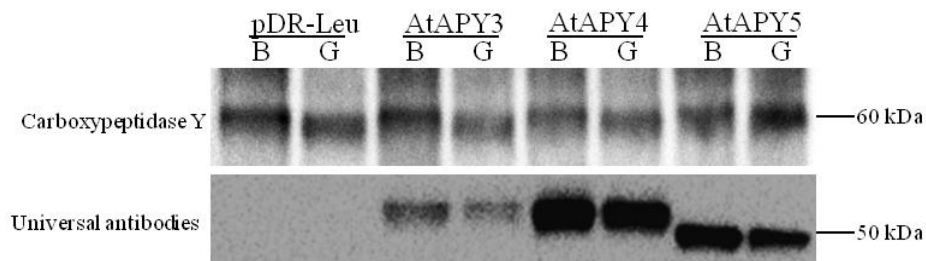


Figure 3.5. Complementation of Golgi-localized yeast GDA with AtAPY3-5.

(A) The heterologous expression of AtAPY5 is capable of rescuing the glycosylation defect in Carboxypeptidase Y, as demonstrated by a mobility shift in the *Δgda1* yeast mutants. Expression of AtAPY3-5 could be assessed using the Universal antibody against a Gateway-specific epitope (attB2) at the C-terminus of the construct.

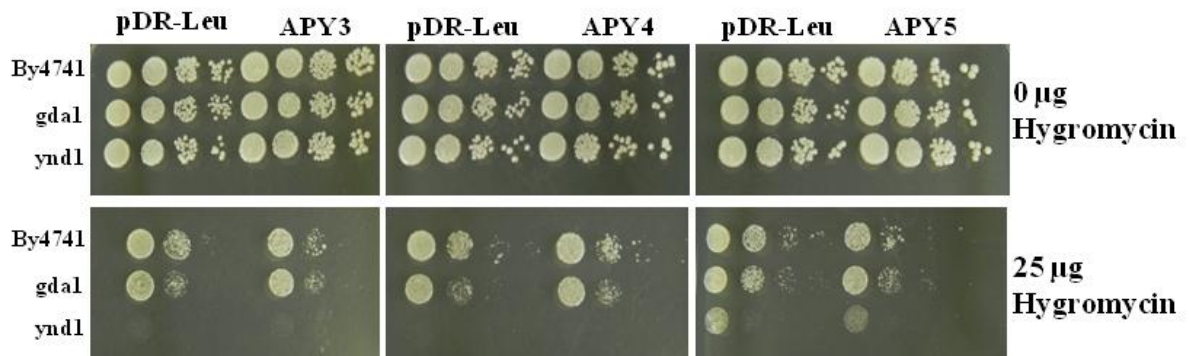
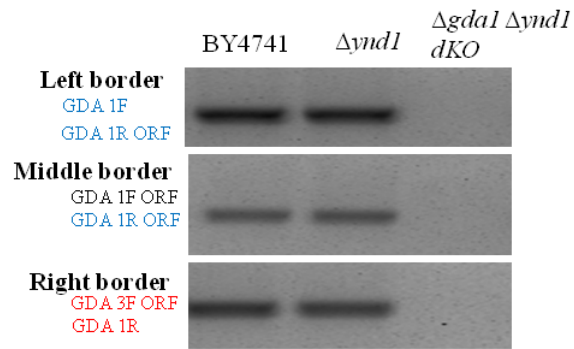


Figure 3.6. Complementation of Golgi-localized yeast NTPDase orthologue YND or GDA with AtAPY3-5. The heterologous expression of AtAPY3-5 in BY4741, *Δgda1* and *Δynd1*. Yeast cells hosting pDR-Leu empty vectors were used as control. The expression of none of AtAPY3-5 can rescue hygromycin sensitivity in *Δynd1* mutant.

A



B

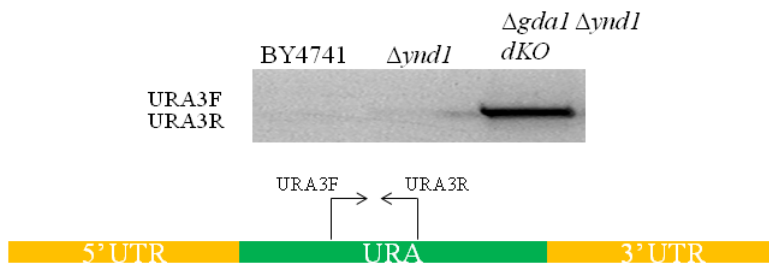


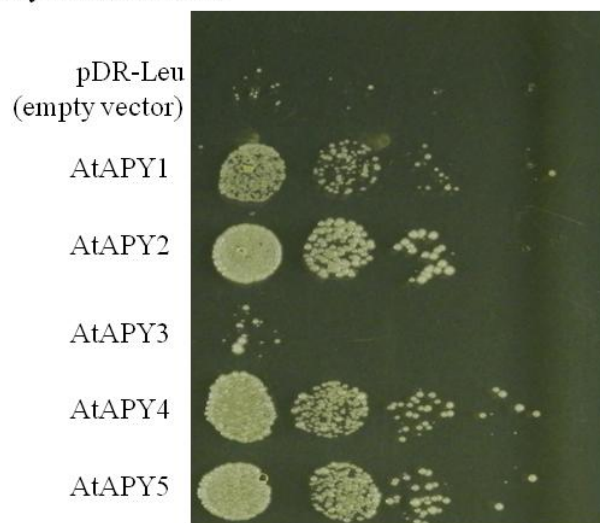
Figure 3.7. Genetic characterization and isolation of $\Delta gda1 \Delta ynd1$ dKO yeast.

(A) PCR screen of transformed $\Delta gda1 \Delta ynd1$ dKO yeast that does not carry the genomic *GDA1* ORF.

(B) PCR screen of transformed $\Delta gda1 \Delta ynd1$ dKO yeast that carries the inserted genomic *URA3* ORF.

A

Δgda1 Δynd1 dKO with



B

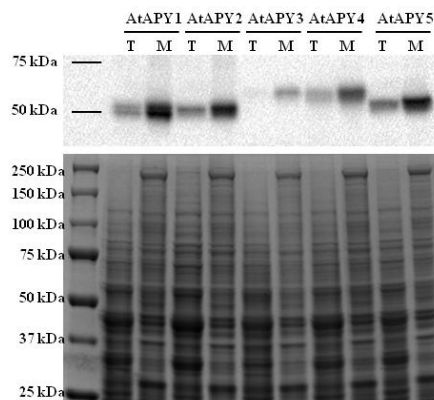


Figure 3.8. Expression of Arabidopsis APYRASEs can rescue the slow growth of *Δgda1 Δynd1 dKO*.

(A) The slow growth of *Δgda1 Δynd1 dKO* can be complemented by expressing Arabidopsis APYRASEs 1, 2, 4, and 5, but not *AtAPY3*.

(B) Immunoblot analysis of *Δgda1 Δynd1 dKO* lines containing *AtAPY1-5* constructs. Total protein (T) and microsomal fractions (M) were isolated from *Δgda1 Δynd1 dKO* harboring *AtAPY1-5* expression vectors. Protein lysates (20 μg) were analyzed by immunoblotting using a universal antibody described in the material and methods.

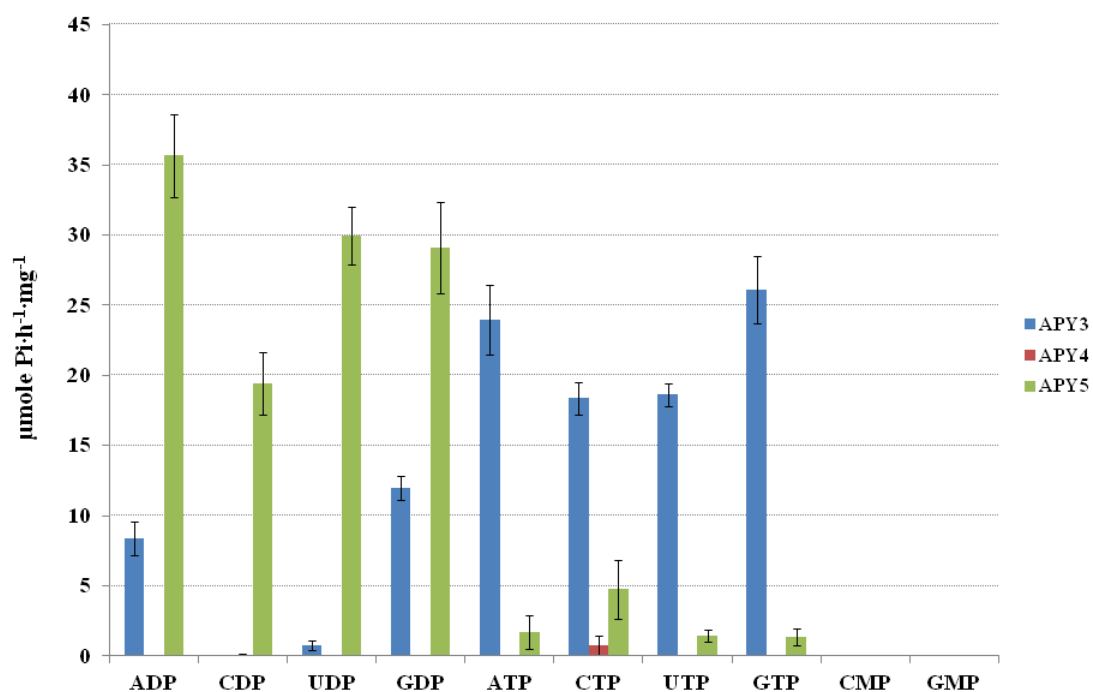


Figure 3.9. Enzyme activity assay of AtAPY3, 4 and 5.

Microsomes were isolated from $\Delta gda1 \Delta ynd1 dKO$ yeast expressing AtAPY3, 4 or 5.

The enzyme activity ($\mu\text{mole Pi h}^{-1} \text{mg}^{-1}$) was measured after incubation for 60 minutes with various substrates ($n = 3$, mean \pm SE).

Chapter 4. Conclusion

Until now there has not been definitive data on the localization of AtAPY1 and AtAPY2. The first co-localization of AtAPY2 in purified Golgi vesicles was reported by Dunkley et al. in 2004. Between 2003 and 2011, Dr. Sun and Dr. Wu, former graduate students from the Roux lab, had used stably transformed or transiently expressed, 35S-driven AtAPY1-GFP to investigate its localization (Yu Sun dissertation, 2003; Jian Wu dissertation, 2007). The results in both dissertations showed an intracellular signal, although the transient localization results of Wu (2007) also showed a peripheral signal. However, the 35S-driven constructs they used failed to complement the *apy1apy2* dKO, perhaps due to their weak expression in pollen tubes (Wilkinson et al., 1997). Since their constructs could not rescue the phenotype at that time, it raised questions about the accuracy of the signal.

Here, I used AtAPY1 and AtAPY2 fused with C-terminus GFP driven by their native promoter to tackle the problem. AtAPY1-GFP and AtAPY2-GFP rescued the *apy1apy2* dKO successfully, which provided support for its proper localization and function. The intracellular punctate structures co-localized with the cis-Golgi Man49-mCherry markers in the root and in the epidermal cells of the cotyledon. This finding was also supported by a recent Golgi proteomics result (Parsons et al, 2011). Further support came from the results of Schiller et al (2012), who crossed the 35S-driven AtAPY1-GFP lines from Yu Sun (Yu sun dissertation, 2003) with *apy1* (single knockout) plants (*apy1apy1* +/-*apy2* SPIK::AtAPY2). With the aid of SPIK::AtAPY2 construct which AtAPY2 can be specifically express in the pollen stage, the 35S-driven AtAPY1-GFP could support the pollen germination stage and rescue the vegetative defect of the double knockout. In addition, the GFP signal obtained from this rescued line also showed Golgi apparatus-associated signals (Schiller et al., 2012). Furthermore, in order to falsify the possibilities of the bleaching of GFP due to the acidic condition in the extracellular space, Schiller et al. also did immunolabeling but still they did not detect any AtAPY1-GFP associated with plasma membrane or the cell wall (Schiller et al., 2012). These findings raised questions about the specificity of the apyrase antibody used to inhibit pollen tube growth and increase the ATP concentration in the pollen growth medium (Wu

et al., 2007), which were the earlier results that argued for a plasma membrane localization of AtAPY1/AtAPY2. It has been demonstrated that the apyrase antibody can also detect the APYs in other plants such as cotton (Clark et al., 2010) and *Ceratopteris richardii* (Bushart et al., in press).

AtAPY1 and AtAPY2 have been hypothesized to be associated with eATP salvage in the extracellular matrix (Steinebrunner et al., 2000). The purified AtAPY1 and AtAPY2 can hydrolyze ATP and ADP but not AMP (Steinebrunner et al., 2000). In Chapter 2, I have demonstrated that knocking out AtAPY1 or AtAPY2 decreased the luminal UDPase activity in microsomes, but had no significant impact on the ADPase activity. In addition, AtAPY1-GFP and AtAPY2-GFP have functional UDPase/GDPase activities that can rescue the UDPase/GDPase activities in the *apy1apy2 dKO* mutants. I have not tested the impact of knocking out AtAPY1 or AtAPY2 on NTPase activities. However, Schiller et al. (2012) have shown that AtAPY1-GFP has a weak ATPase activity but a much stronger UDPase activity (Schiller et al., 2012). These results raised more questions about how AtAPY1 and AtAPY2 could function to control extracellular nucleotide signaling, and they need to be resolved in the future.

The nucleotide sugars cross the Golgi membrane from the cytosol in an antiporter manner (Reyes and Orellana, 2008). Based on the prior reports about Golgi luminal NDPases associated with glycan synthesis and nucleotide sugar transport in mammalian cells and yeast (Knowles, 2012), I tested the impact of knocking out AtAPY1 and AtAPY2 in plants. However, in the estradiol-induced R2-4A mutant, in which *AtAPY1* is suppressed by RNAi in *apy2* mutants, the UDPase activity still has residual activity that is 50 % of the WT, which may be enough to support the uptake of UDP-galactose measured in vitro. Interestingly, we have observed the only sugar level significantly changed in cell wall material of all mutants analyzed (*apy1*, *apy2* and R2-4A) was galactose, and its level was elevated. Several UDP-galactose transporters have been reported, but only one Golgi-localized transporter (AtNST-KT) has been demonstrated to act as an antiporter (Rollwitz et al., 2006). However, it has also been proposed that a specific type of UDP-galactose transporter may form complexes with UDP-glucose 4-epimerases (UGEs) (Handford, 2006; Seifert, 2004). The flux of UDP-galactose is

regulated by the coupling of the isoforms of UGEs, the cytosolic redox potential (NAD⁺/NADH) and the growth regulators (Handford, 2006; Seifert, 2004).

I have also tested the effect of tunicamycin, a blocker of N-linked glycosylation, on the root structure of loss-of-function mutants of APY1 or APY2 (Fig. 4.1). However, none of the concentrations tested showed significant impacts (Fig. 4.1). In addition, blocking N-linked glycosylation may cause callose deposition in the roots (Lukowitz et al., 2001), but we observed no obvious callose deposition on any mutants, even the *APY1 RNAi apy2* (R24A) (Fig. 4.2). Furthermore, two N-linked glycoproteins, protein disulfide isomerase (PDI) and calreticulin, were examined by immunoblot analysis. Neither of them showed lack of proper glycosylation (Fig. 4.3). These results suggested that N-linked glycosylation was insensitive to the suppression of AtAPY1 and AtAPY2. Up to now, it is still unclear what functions AtAPY1 and AtAPY2 have in the Golgi lumen and how their enzyme activities can be regulated there.

In past decades, the Roux lab put more efforts on the functional characterization of AtAPY1 and AtAPY2 of regulating plant growth and development. Less attention was paid to the other AtAPY members in Arabidopsis until Jian Yang carried out initial functional characterizations of AtAPY3-7, and found that AtAPY6 and AtAPY7 may also play roles in the ER/Golgi that could involve glycan synthesis (Jian Yang dissertation, 2011). He did not obtain any physiological information on the function(s) of AtAPY3-5 from his mutant analyses. This lack of phenotypes was hypothesized to be due to the functional redundancy among them. Here, I expressed these AtAPYs in the onion epidermal cells and yeast and obtained some of preliminary localization and biochemical characteristics of them.

The localization, biochemical analysis and complementation assays showed that AtAPY3-5 have roles distinct from each other. AtAPY3 is non cis-Golgi localized and has preferences toward NTPs which cannot rescue the defect caused by the loss of Golgi luminal NTPDase GDA and YND in yeast. AtAPY4 seems to be a weak AtAPY that plays some roles in the cis-Golgi since it can still partially complement both the N-linked glycosylation activity in GDA and the slow growth phenotypes in $\Delta gda1 \Delta ynd1$ *dKO*. AtAPY5 is a much stronger NDPase. It prefers a wider range of NDP substrates. This also coincides with its role in rescuing yeasts Golgi luminal NTPDase mutants.

Transiently expressed AtAPY6-YFP and AtAPY7-YFP in the onion epidermal cells showed ER, and cis-Golgi localization, respectively. This piece of evidence supports possible mechanism of causing pollen cell wall defect observed in *apy6 apy7 dKO* by Dr. Yang (Jian Yang dissertation, 2011). However, the heterologously expressed AtAPY6 and AtAPY7 can not be detected, which hinders the further biochemical analysis.

My dissertation resolved some of the arguments about the subcellular localization and the possible intracellular roles of AtAPY1 and AtAPY2 in Arabidopsis. It also provides biochemical and transient localization information of other Arabidopsis APYRASE members to aid future investigations of their physiological roles in plants.

Key remaining questions

As I described in the chapter 1, the pH of the plant extracellular matrix is more acidic (pH5.5) compared to what it is in mammalian cells (pH7-8). However, the best pH for the NTPDases (APYRASEs) to function is close to neutral. The ADPase activity of Ynd1 decreases sharply with decreasing pH. The ADPase activity is minimal when the pH drops to 6 (Zhong et al., 2000). Although up to now, four papers (Dunkley et al., 2004; Parsons et al., 2012; Schiller et al., 2012; Chiu et al., 2012) indicate that AtAPY1 and AtAPY2 are Golgi luminal NTPDases by various methods, the biochemical function of these APYRASEs is still not completely resolved as to whether it functions only intracellularly or both intra-and extracellularly. It will be interesting to know in the future what is the environmental pH range in which these Arabidopsis APYRASEs function, for this information will provide some additional evidence on their subcellular localization.

A second major unanswered question concerns the function of AtAPY3. This member of the family seems to be a distinct APYRASE based on the transient localization data and the yeast double knockout complementation assay. In addition, it has the enzyme activity appropriate for it functioning as an ecto-NTPDase. Further studies are need to discover the physiological significance of this apyrase in Arabidopsis.

Appendices

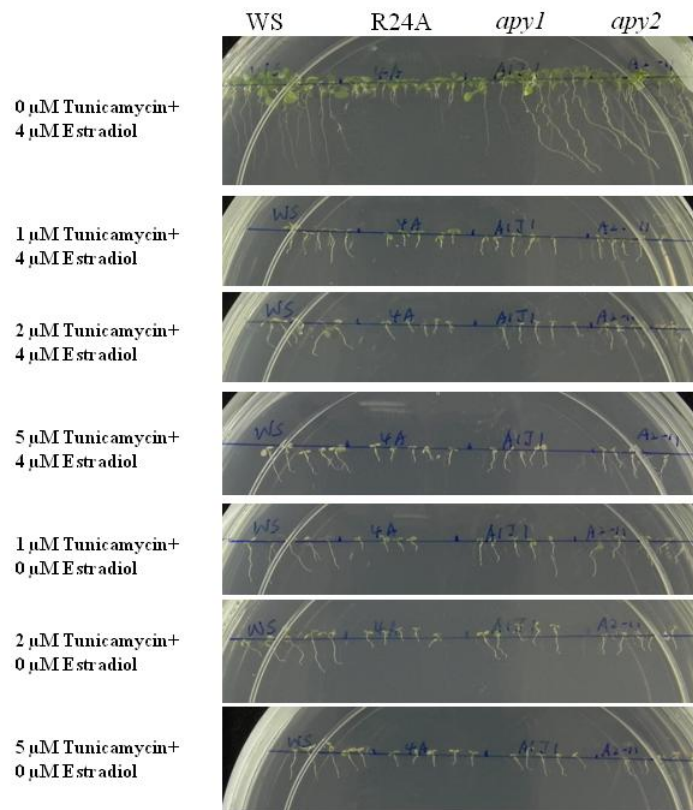


Figure 4.1. Effects of tunicamycin on APYRASE mutant roots. The 4 μ M estradiol was used to induce the RNAi construct as the R24A control. None of the APYRASE mutants root showed any sensitivity to the tunicamycin when compared to the wildtype (WS).

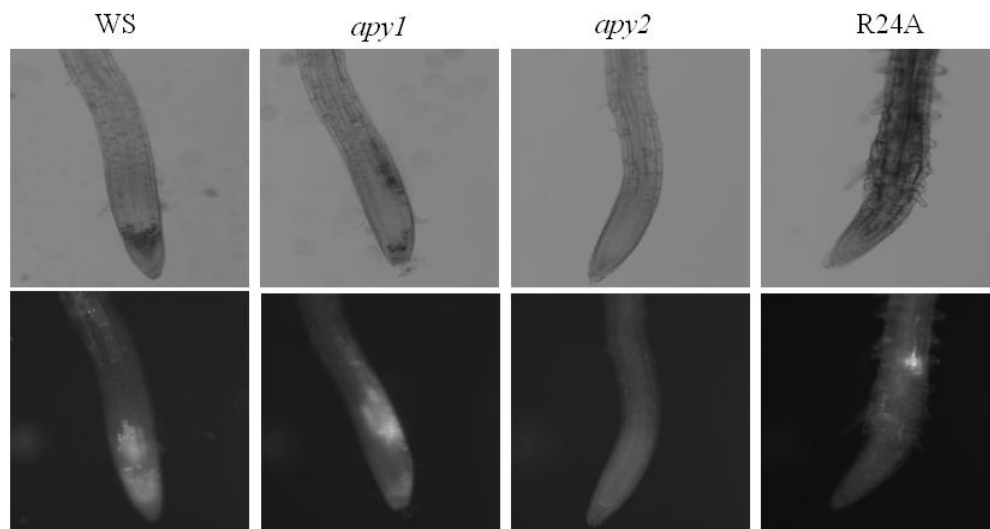


Figure 4.2. Effects of knocking out APYRASEs on callose deposition of the mutant roots. Bright field (upper panel) and epifluorescence images (bottom panel) of Arabidopsis root tips stained with the callose-specific fluorescent dye aniline blue.

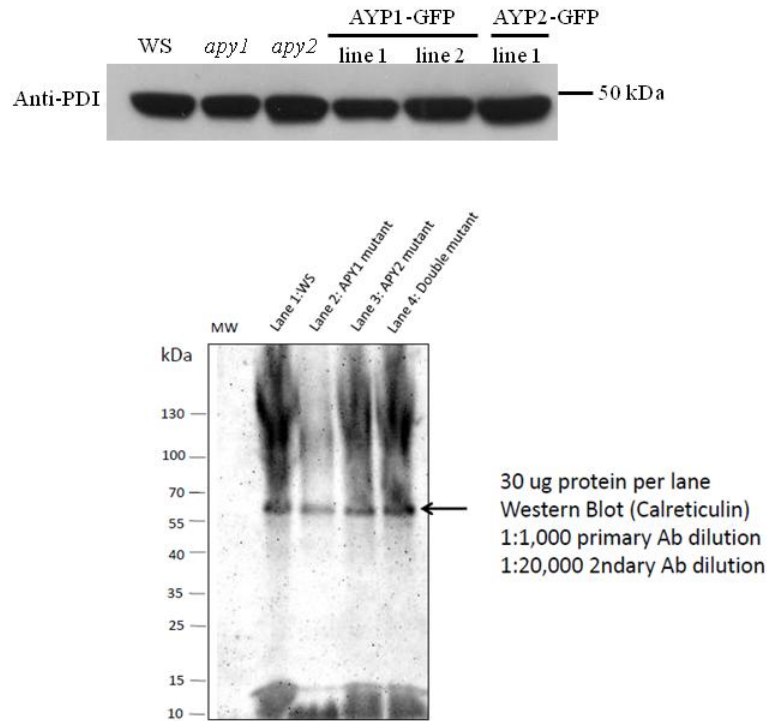


Figure 4.3. Immunoblot analysis of N-link glycosylation markers in APYRASEs mutants

(A) Total protein (20 μ g) isolated from APYRASEs mutants were probed using antibody to human protein disulfide isomerase (PDI) (Genescript).

(B) Total microsomal protein (20 μ g) isolated from APYRASEs mutants were probed using antibody to calreticulin. The blot is kindly provided by Dr. Ito from JBEI.

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Vita

Tsan Yu Chiu graduated from National Taiwan University in Taipei, Taiwan in May, 2003. She obtained the degree of Master of Science from University of British Columbia, Canada in December, 2006. In May, 2008, she began her Ph.D. journey at The University of Texas at Austin, USA.

Permanent address: sonyachiu@utexas.edu

This dissertation was typed by the author.